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Irene Helen. Demsar  
*University of Windsor*

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THE EFFECTS OF ULTRAVIOLET LIGHT ON SPORE GERMINATION  
IN THE CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

by



Irene Helen Demsar

A Thesis  
submitted to the Faculty of Graduate Studies  
through the Department of Biology  
in Partial Fulfillment of  
the requirements for the  
Degree of Master of Science at  
the University of Windsor

Windsor, Ontario, Canada  
1981

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1981

**763005**

DEDICATION

This thesis is dedicated to my late father  
who had a keen interest in science  
and advocated the pursuit of  
knowledge and truth in life

ABSTRACT

THE EFFECTS OF ULTRAVIOLET LIGHT ON SPORE GERMINATION  
IN THE CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

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The spore germination process in Dictyostelium discoideum is a model system with which to study gene expression in a simple eukaryote. Spore germination has been divided into the following four stages: activation, post-activation lag, swelling and emergence. Ultraviolet irradiation ( $250 \text{ J/m}^2$ ) of spores at any time prior to late spore swelling allows full swelling, but inhibits the emergence of myxamoebae. A UV exposure time of 30 seconds ( $50 \text{ J/m}^2$ ) is sufficient to reduce emergence to about 6 % when measured after 24 hours of incubation. This same fluence results in about 10 % viability as measured by plaque forming ability. Experiments utilizing fractionated exposures or exposures to lower UV fluence rates ( $0.5 \text{ J/m}^2/\text{sec}$ ) result in the same percentage inhibition of emergence as that found for single UV exposures at  $1.7 \text{ J/m}^2/\text{sec}$ , provided the total fluence is equivalent. Ultraviolet irradiation after late spore swelling allows emergence to occur in only a small fraction of the population. This fraction of cells which emerges after UV treatment is said to have passed a critical point which we have defined as the "competence point". The competence point is believed to be the time when all the necessary events for emergence have been completed.

The higher UV fluences ( $250 \text{ J/m}^2$ ) which completely prevent emergence, do not affect the endogenous oxygen uptake of spores during swelling. Ultraviolet light irradiated spores respond to the same activation and deactivation treatments as control non-irradiated spores. The phenomenon of autoactivation of Sgl spores is not prevented by UV irradiation.

UV irradiation ( $250 \text{ J/m}^2$ ) immediately after heat activation does however, inhibit the stage specific accumulation of radiolabelled uracil into germinating spores. A UV exposure time of 45 seconds ( $77 \text{ J/m}^2$ ) which reduced the final percent emergence to below 1 %, also inhibits uracil incorporation to a level only about 10 % that of control values. Consequently protein synthesis, as reflected by the stage specific accumulation of radiolabelled leucine into germinating spores, is inhibited in irradiated spores to the same degree as cycloheximide treated spores. However, the incubation of spores in the presence of cycloheximide also reduced the level of uracil which is incorporated during germination. Furthermore, cycloheximide treated spores do not release myxamoebae if they are irradiated within 1 hour after the cycloheximide has been removed. DNA synthesis, as determined by the incorporation of radiolabelled thymidine could not be detected in germinating spores. There was also no evidence of unscheduled DNA repair synthesis in irradiated spores.

Macromolecular synthesis in spores was also evaluated by observing the effect of UV upon the stage specific expression of three enzymes. The normal accumulation of B-glucosidase,



alkaline phosphatase and trehalase specific activities were inhibited in UV irradiated spores. The bulk of the specific activities of all three enzymes are expressed after the competence point has already been achieved in germinating spores. It thus seems possible that these three enzymes may not have a critical function in the emergence process. The enzymes which are potentially essential to the emergence process would have to be synthesized prior to the competence point, and possible during early and middle swelling. We do not exclude the possibility that some essential enzymes may be a result of preformed message stored in the dormant spore.

We believe that the inhibition of macromolecular synthesis in UV irradiated spores is due to the presence of unrepaired UV-induced pyrimidine dimers in the DNA template. Though the site(s) of UV inactivation in Dictyostellium discoideum spores can only be postulated at present, it is apparent that the initial events in germination (reversible activation, swelling and respiration) occur independently of the UV sensitive site(s). The final event (emergence) however, is dependent on a UV sensitive function.

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## ABBREVIATIONS

CPM	Counts per minute
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ergs/mm <sup>2</sup>	Ergs per square millimeter
J/m <sup>2</sup>	Joules per square meter
KH <sub>2</sub> PO <sub>4</sub>	Potassium phosphate
MgSO <sub>4</sub>	Magnesium sulfate
mRNA	Messenger ribonucleic acid
Na <sub>2</sub> HPO <sub>4</sub>	Sodium phosphate
NH <sub>4</sub> Cl	Ammonium chloride
O <sub>2</sub>	Oxygen
PO <sub>4</sub> buffer	Phosphate buffer
rpm	Rotations per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
TCA	Trichloro acetic acid
TMV	Tobacco mosaic virus
tRNA	Transfer ribonucleic acid
UV	Ultraviolet

## INTRODUCTION

Dictyostelium discoideum is a primitive eukaryotic organism which was originally isolated from decaying forest matter in 1935 by Dr. K. B. Raper. Since that time the organism has become a major research tool for the study of basic life processes such as intracellular and multicellular development (Bonner, 1967; Loomis, 1975). In addition, the organism is rather radiation resistant and thus has been useful in the study of cellular repair mechanisms (Deering, 1975).

Much of the present knowledge concerning the regulatory processes which are operating in this organism was obtained by subjecting cells to various chemical and physical stresses. The significance of the studies using stress techniques will be discussed in the literature review section of this thesis. Relevant studies in other biological systems will also be considered throughout, in order to set the precedent for the final conclusions of the thesis.

The body of this thesis will focus on the spore germination process of Dictyostelium discoideum. This includes a further elucidation of the macromolecular events that are required for the transition from a quiescent spore structure to an actively growing cell. More specifically, the approach

of using a physical inhibitor (ultraviolet light) is introduced as a useful technique with which to probe macromolecular synthesis in the germinating spore. It is hoped that the studies presented in this thesis, in conjunction with earlier studies, will contribute to our basic understanding of the regulation of intracellular development in Dictyostelium discoideum and higher eukaryotes as well.

## LITERATURE REVIEW

### 1. The Developmental Life Cycle of Dictyostelium discoideum

Much attention has been focused on the developmental aspects of the life cycle of Dictyostelium discoideum. The life cycle of this organism is composed of several contiguous stages. There is first the vegetative ameboid growth phase during which cells reproduce by binary fission. These vegetative cells can be maintained in the logarithmic phase of growth in the presence of a bacterial food source. Starvation conditions, however, trigger the initiation of the multicellular developmental phases (Bonner, 1967; Newell, 1978a, b). During early development, cell aggregation occurs in response to the secretion of cyclic AMP by an initially small number of cells in the population (Konijn and Raper, 1961; Konijn et al., 1968; Robertson and Grutsch, 1974). Migrating slugs are then formed by the aggregating streams of amoebae. As development continues the mass of cells in the slug differentiate into two basic cell types which are designated prestalk cells and prespore cells. The formation of the fruiting body containing spores is the terminal stage of the developmental cycle. The fruiting body at some later time can topple over to disperse the spores; in response to the presence of food (or other activating conditions) the spores can germinate to release amoebae (Cotter, 1981).

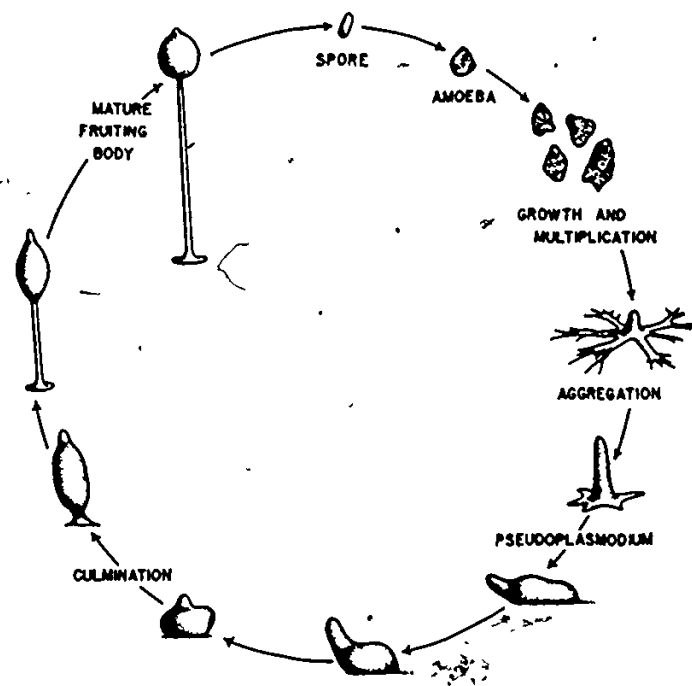


Figure 1. The life cycle of Dictyostelium discoideum

It is the processes which occur during developmental differentiation which have been of major concern in the study of this organism. The sequence of events which occur at this time have been postulated by some researchers to be a result of differential gene expression (Roth et al., 1968; Loomis, 1969b; Firtel, 1972; Jacobson and Lodish, 1975). The specific activities of a number of enzymes have been observed to change significantly during development (see Loomis, 1975 for a review). Chemical inhibitors of RNA and protein synthesis have been employed in attempts to discern whether the changes in specific activity of these enzymes are a result of de novo synthesis, and thus represent alterations in specific gene activity. It was concluded by these researchers that genes were transcribed and translated sequentially, according to a developmental program (Loomis, 1975). However, it has been cautioned by other researchers (Quance and Ashworth, 1972) that the theory of selective gene activation may only provide a partial explanation of regulation during the differentiation process. This view has been shared by Gustafson and Wright (1972) who also suggest that consideration must be given to possible allosteric regulation exerted by varying metabolite levels. Gustafson and Wright further point out that the protein turnover rate must also be considered before one can attribute the changes in specific activity to actually represent changes in the rate of enzyme synthesis itself. In general, the increase in enzyme specific activity

can be the result of the following causes as outlined by Every and Ashworth (1975b):

1. The activation of pre-existing enzymes by diffusible activators.
2. The removal of diffusible inhibitors of enzyme activity.
3. The removal of non-diffusible "masking" substances bound to the enzyme.
4. A decrease of enzyme degradation against a background of steady enzyme synthesis.
5. A conversion of an inactive precursor enzyme.

A number of "stage specific" enzymes, all of which have been shown to require concomitant protein synthesis and a prior period of RNA synthesis have been examined by Loomis (1975). He correlated a lack of specific enzyme expression in a number of isolated developmental mutants with their specific morphological limitations. This would add support to the idea of the requirement for synthesis of certain enzymes for the completion of morphological events. In general, enzymes such as those examined by Loomis, are considered to be developmentally regulated if they follow the criteria as outlined by Quance and Ashworth (1972):

1. The specific activity rises significantly (more than threefold).
2. This increase in specific activity is aberrant in morphologically deranged mutants.

3. This increase in specific activity is affected by known inhibitors of protein synthesis.
4. This increase in specific activity is affected by mechanical perturbation of the normal morphogenetic sequence.

Early work with actinomycin D as an RNA synthesis inhibitor attempted to correlate the accumulation of some enzymes with the differential rate of mRNA synthesis during a specific stage (Sussman, 1966, 1967). Other workers have since employed a combination of actinomycin D and daunomycin, to effect a more complete inhibition of both mRNA and rRNA to 98%, and inhibited the biosynthesis of three developmentally regulated enzymes as well (Firtel et al., 1973). It was thus hypothesized that the primary control of protein biosynthesis during Dictyostelium discoideum vegetative development was at the level of mRNA transcription (Firtel et al., 1973). A number of workers have recently demonstrated transcriptional control of gene expression during Dictyostelium vegetative development by examining the pattern of proteins synthesized at different stages of differentiation by two dimensional polyacrylamide gel electrophoresis, and comparing it to isolated mRNA whose translated products were similarly subjected to gel analysis (Alton and Lodish, 1977a, 1977b; Dowbenko and Ennis, 1980). They concluded that "the major control mechanisms regulating the pattern of protein synthesis operate at the level of either



transcription of the gene, processing of the transcripts to a translatable form, or degradation of the mRNA." (Alton and Lodish, 1977).

Thus, there have been demonstrations of transcriptional regulation occurring during the developmental phase of the life cycle of Dictyostelium discoideum. However, there is still little known about other levels of regulation that may also be occurring at this time.

## 2. The Spore Germination Process of Dictyostelium discoideum

The process of spore germination in Dictyostelium discoideum is also a developmental process and has been described as a suitable system for studying the regulation of gene expression. Developmentally regulated changes in both protein and mRNA syntheses occur during the transition from the dormant spore to the growing amoeba (Dowbenko and Ennis, 1980). The macromolecular synthesis associated with the conversion from the dormant state to the actively growing phase has been examined by a number of researchers. Both protein and RNA synthesis, as measured by the incorporation of radiolabelled precursors, have been shown to occur during spore germination (Bacon and Sussman, 1973; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977, 1978). These researchers have shown that chemical inhibitors of protein synthesis block the normal accumulation of radiolabelled amino acids during spore germination. Furthermore, the accumulation of the "stage

specific" expression of a number of enzymes, which normally occurs during spore germination, is also blocked by inhibitors of protein synthesis (Cotter and Raper, 1970; Cotter et al., 1979, Tisa and Cotter, 1980). Finally, when activated spores are incubated in the presence of protein synthesis inhibitors such as cycloheximide, they swell normally, but do not release amoebae (Cotter and Raper, 1970). It has thus been suggested that protein synthesis is required for the emergence stage of germination (Cotter and Raper, 1970; Tisa and Cotter, 1980). Evidence for the specific level of regulation of the synthesis of proteins during spore germination has recently been obtained. Dowbenko and Ennis (1980) have found that mRNA which is isolated from spores at different stages of germination and translated in a wheat germ cell-free protein synthesizing system produced the same protein patterns in two-dimensional polyacrylamide gels as did the proteins that were synthesized in vivo. This they suggested was an indication that regulation of protein synthesis during spore germination was primarily at the level of production and destruction of mRNA. This attractive postulation has not yet been firmly established however. It is not yet certain whether the protein synthesis required for the emergence stage of germination is entirely dependent on "de novo" RNA synthesis since the dormant spore contains preformed mRNA (Bacon and Sussman, 1973; Giri and Ennis, 1980; MacLeod et al., 1980). Some chemical inhibitors of RNA synthesis inhibit the normal

accumulation of radiolabelled precursors of RNA during spore germination and also block the emergence stage of germination (Giri and Ennis, 1977); however, they also block the swelling stage of germination under some conditions (Giri and Ennis, 1977; Hamer and Cotter unpublished results).

Thus, there may be other unknown side effects when certain chemical inhibitors are used to block RNA synthesis.

It is frequently difficult to ascertain the mechanism of inhibition of RNA synthesis utilizing inhibitors such as daunomycin, thiolutin, lomofungin and actinomycin D since it is always possible that the agent used in one system acts differently in another system. For example, actinomycin D, which does not block the swelling stage of germination also does not completely inhibit emergence in Dictyostelium discoideum spores (Cotter and Raper, 1966; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977). Yagura and Iwabuchi (1976) used actinomycin D (60 ug/ml) as an RNA synthesis inhibitor and concluded from their sedimentation patterns that only ribosomal RNA synthesis was inhibited and not the heterodisperse RNA or the 4-5s RNA. This is similar to the actinomycin D effect observed for vegetative cells by Firtel and co-workers (1973). Yagura and Iwabuchi (1976) tentatively concluded that mRNA synthesis (which was resistant to actinomycin D) may be involved in the synthesis of proteins required for spore germination. However, Giri and Ennis (1977) point out that it could not be proven whether actinomycin D could

effectively permeate the spores, and thus the hypothesis proposed by Yagura and Iwabuchi is not well supported.

Indeed, it is apparent that the entire line of evidence for the RNA synthetic requirements for emergence remain somewhat inconclusive thus far. An effective inhibitor of RNA synthesis which also completely blocks only the emergence stage of germination would be required to provide more concrete evidence.

A very well characterized inhibitor of macromolecular synthesis that has been employed in many systems is ultraviolet light. The action of this physical agent and its potential as an inhibitor of RNA synthesis will be considered in the following paragraphs of this literature review.

### 3. The Effects of Ultraviolet Light on Macromolecular Synthesis

Light in the ultraviolet range of the electromagnetic spectrum has been implicated in the induction of "biologically significant" lesions. It has been well documented that far UV light (254 nm) in particular is absorbed by and causes modifications of the nucleic acids (Jagger, 1967). Of these UV-induced modifications, one of the most well characterized is the pyrimidine dimer (Setlow, 1966). It has been demonstrated that the presence of such dimers in a polynucleotide strand can affect template function such that there is a delay or blockage of the progress of DNA and RNA polymerases along the DNA template (Michalke and Bremer, 1969). This results

in an inhibition of DNA synthesis required for the replication process of cells as well as a termination of transcription of RNA at the sites of unrepaired dimers along the template (Michaelke and Bremer, 1969; Sauerbier et al., 1970; Sauerbier, 1976). The consequence of the resulting shortened messenger RNA molecules containing incomplete information is a respective reduction of protein synthesis as well as the synthesis of abnormal proteins (Brunschede and Bremer, 1969). The fact that there is an inhibition in the induced synthesis of enzymes of irradiated cells supports the idea that this inhibition is a result of a decrease in the rate of transcription which is related to the radiation damage of the DNA (Kameyama and Novelli, 1962; Swenson and Setlow, 1964; Pardee and Prestidge, 1967).

The transcription terminating effects of UV light that were originally studied in E. coli and other bacterial cells have since been extended to eukaryotic cells. Hackett and Sauerbier (1974) concluded that premature termination of transcription is the major effect of UV irradiation on transcription of the L cell genome and that most of the transcription terminating UV photoproducts are pyrimidine dimers. It has been observed that such transcription terminating lesions exert a strong polar effect on the expression of promotor distal portions of monocistronic and polycistronic transcription units (Sauerbier and Hercules, 1978). This phenomenon is the basis on which the technique of "UV mapping" was developed in E. coli and has since been used in *Drosophila*,

mouse L cells, and hela cells (Carlson et al., 1977; Hackett and Sauerbier, 1975; Goldberg et al., 1977). Kantor and Hull (1979) found that UV caused an immediate and equal depression of RNA synthesis in both repair proficient and deficient non-dividing human diploid fibroblasts. This is also consistent with the hypothesis that the lethal action of UV in non-dividing cells is one on DNA, that leads to the inhibition of protein synthesis by preventing RNA transcription. In general, it has been stated that in dividing cells, the biological consequences of DNA damage is cell inactivation and probably reproductive cell death. In non-dividing cells, the consequences of DNA damage are impaired genetic transcription and thus translation, leading to the suppression of the synthesis of enzymes (Kanazir, 1969).

Given this myriad of evidence of the transcription terminating effects of UV light, it is not hard to see how such a physical agent may be an "ideal" tool to probe macromolecular synthesis in a system in which various chemical agents may be presented with permeability barriers. However, researchers have reported effects of UV on certain physiological processes such as respiration and membrane transport in some systems, which depend on the specific wavelength and the intensity of the UV fluence (Doyle and Kubitschek, 1976). Under certain conditions, UV may also induce a number of non-dimer effects as well (see Rahn, 1979, for a review).

For example, in eukaryotic cells, there is a greater chance of UV-induced DNA-protein crosslinks, due to the high histone content (Smith, 1968, 1976). Thus it is important to examine such parameters in irradiated systems to ensure that the UV fluence used is not causing other unintended anomalies. Since the RNA of cells is also nucleic acid in nature, it must also be considered as a potential site of UV susceptibility. The question of UV effects on translation have ~~been~~ examined by some researchers. Though there have been reports of damage to the mRNA and translation apparatus in UV irradiated E. coli (Swenson and Setlow, 1964), it was concluded that the structural gene was the major target of UV, and thus DNA was the major target for loss of viability in irradiated cells, although it was not the only one (Pardee and Prestidge, 1966). In general it appears that translation functions are apparently less sensitive than the transcription requiring events. Atsuti et al., (1964) examined the effect of UV irradiation on the induced synthesis of catechol oxygenase in Pseudomonas effusa and concluded that the inhibition of the induced formation of the enzyme was primarily a result of damage to the DNA template rather than to an effect on template activity of mRNA. Indeed, meaningless information was transcribed into mRNA from UV-damaged DNA (Nozu et al., 1965). Thus the process in which mRNA, tRNA, and ribosomes were involved in the formation of enzyme (translation) did not appear to be UV sensitive. The UV sensitivity of TMV RNA infectivity, which requires RNA replication, is about eight fold greater than the sensitivity of the translational

activity of TMV RNA (Murphy, 1973). Similarly, it was found that the quantum yield for the loss of infectivity of R 17 virus (an RNA bacteriophage) was 5-10 times higher than the quantum yields for the destruction of the translation of the RNA into required protein (Yamada et al., 1973). Such work lends support to the hypothesis that mRNA (preformed, or once transcribed) is not as greatly affected by UV as is the transcription process. However, in a recent study on four experimental systems lacking DNA repair, Helmut and co-workers (1979) found that a unit length of mRNA (in bases) is 2-3 times more sensitive than a unit length of DNA (in base pairs) with respect to inactivation of template function. Nevertheless, in any system there will be many more molecules of mRNA which would require inactivation compared to the single DNA macromolecule, and it would seem that any observed inhibition of gene expression would still be primarily due to DNA template inactivation. Even with the previous discussion in mind, the possible effects of UV on the translation process should not be overlooked when conclusions are being finalized for any particular irradiated system.

In addition to the above mentioned parameters which should be examined in irradiated systems, it is also important to screen the UV repair capabilities of the cells in question before reaching conclusions on the UV effects of macromolecular synthesis. The usefulness of UV light as a probe for molecular studies can be diminished in organisms with very efficient mechanisms of DNA repair. The next portion of this



literature review will be devoted to a description of the repair capacities of Dictyostelium discoideum cells with particular emphasis on the sensitivities of the spores. It will become apparent that UV light can be uniquely applied to the study of spore germination as opposed to the study of vegetative cells which have efficient mechanisms of repair.

#### 4. Repair Capacities of Dictyostelium discoideum cells

Most living organisms have a number of capacities which allow them to survive and evolve (Cleaver, 1978). The major systems for DNA repair which include photoreactivation, excision repair, and recombinational repair, were originally discovered and defined in bacteria. They have since been observed to occur in eukaryotic cells as well. However, some of the mechanisms of repair were described as having very different forms and functions in eukaryotic cells. Cleaver (1968) stresses that the greater complexity of the DNA structure and organization in eukaryotic cells must be considered in attempts to explain the responses of such cells to irradiation.

There have been a number of studies in recent years in which the capabilities of DNA repair of the lower eukaryote, Dictyostelium discoideum, have been examined. Much of the research to date has been involved in defining the repair capacities of the relatively resistant vegetative cells of this organism. Deering (1968) found Dictyostelium discoideum

vegetative cells capable of surviving extensive ionizing gamma radiation, and described this slime mold to be among the most radiation resistant organisms known. The responses of the vegetative cells of this organism to ultraviolet radiation were also examined by Freim and Deering (1970). They concluded that the dark repair mechanisms were so efficient as to mask a possible photorepair mechanism. Photorepair was only apparent when cells were incubated in the presence of caffeine, a known inhibitor of dark repair in bacterial and eukaryotic systems (Harm, 1967; Lehman and Kirkbell, 1972). The dark repair also seemed to operate in the correction of damage due to alkylating agents (Payez et al., 1972).

Additional evidence for the presence of dark repair mechanisms was presented by Khoury and Deering (1973) who observed that the enhanced survival of the NC4 strain appeared to be correlated with the ability to repair single strand breaks in DNA. However, two gamma sensitive strains ( $\gamma$ -sl3) and  $\gamma$ -sl8) that had been isolated by this time, also seemed capable of rejoining single strand breaks induced by gamma rays. In the case of UV irradiation Guialis and Deering (1976) also observed that single strand breaks were induced in UV irradiated strains of both NC4 and the  $\gamma$ -sl3. Nevertheless, Guialis and Deering deduced that UV did not directly induce single-strand breaks as was the case for ionizing gamma radiation, but rather, the induction of single strand breaks by UV was an enzyme mediated process. This idea was supported

by the fact that an endonuclease had been detected in the homogenates of Dictyostelium discoideum cells by Deering and Jensen (1975) which was active in producing single strand breaks in irradiated  $\phi$ X174 RF1 DNA. The endonuclease was apparently acting at the site of UV-induced lesions, most probably pyrimidine dimers. Guialis and Deering (1976) estimated that a fluence of  $100 \text{ J/m}^2$  (of 265 nm UV) resulted in about 0.3 per cent thymine containing dimers relative to total thymine. In this study, Guialis and Deering also compared the fate of UV-induced thymine containing dimers in the NC4 and  $\gamma$ -sl3 strains. It was found that NC4 required  $200 \text{ J/m}^2$  (of 265 nm UV) to reduce survival to the 10 per cent level, while the  $\gamma$ -sl3 required less than  $1 \text{ J/m}^2$  to exhibit comparable reduced survival. The dimers, however, were found to be effectively excised in both the wild type and the  $\gamma$ -sl3 strain. It is found that the  $\gamma$ -sl3 strain was not defective in the incision, excision or rejoining steps of dark repair, and its extreme sensitivity would have to be attributed to some other defect that acted after the excision repair steps. It was postulated that  $\gamma$ -sl3 might even have a defect in the coordination between the enzymes of repair and those of normal DNA synthesis (Guialis and Deering, 1976). Other less sensitive mutants had also been isolated by Deering, and eventually a number of repair deficient mutants had been further characterized, and their recessive mutations could be assigned to particular loci (Welker and Deering, 1978a, b, 1979). The  $\gamma$ -sl8 mutant for example was characterized to have a

mutation at the Rad C location. Rad C mutants were defective in the enzymatic production of single strand breaks associated with excision repair. Since both radiation resistance and sensitive strains with a mutation at the Rad C locus were able to repair most of the single strand breaks in their DNA, it was concluded that the rad C locus affected the excision repair of UV damaged DNA, probably by affecting a "UV-damage specific" endonuclease. Other mutation loci that were found included the Rad A and Rad B mutations. Such mutants were even more sensitive than the Rad C mutants. Unlike Rad C mutants, which exhibited a slight shoulder in their survival curves, Rad A and B mutants exhibited no shoulder. The Y-s13 mutant fell into this category of mutants. Welker and Deering (1979a) demonstrated that the Rad A and B gene products were involved in a common repair pathway which was different from Rad C gene products. They postulated that Rad A and B gene products may be involved in some type of post-replication repair. More recently, Kielman and Deering (1980) attempted to further define the Rad B mutation which appeared to render the cells "super-sensitive" to UV. Unlike the Rad C mutation in which a delay in semi-conservative replication and cell division is commonly observed after irradiation, the Rad B mutant did not exhibit much of a decline in DNA synthesis after irradiation (Kielman and Deering, 1980). It was reasoned that for the Rad C mutant, unexcised UV-induced pyrimidine dimers were effectively blocking the progress of polymerases along the DNA template. Thus, semi-conservative DNA

replication did not resume along the DNA template until much of the damage had been repaired. They thus postulated that the Rad B mutation might be an altered 3'-5' exonuclease proof-reading and/or polymerase function allowing incorrect bases to be inserted opposite damaged parental strands with little blockage of polymerase progression along the template. Most recently, Clark and Deering (1981) reexamined the fate of UV induced pyrimidine dimers in NC4 cells and in a Rad C mutant with a sensitive endonuclease assay. They found that the Rad C mutant was capable of removing dimers but the mutant removed the dimers at a much slower rate than the NC4 strain. Their results were still consistent with the fact that the mutant was deficient in the activities responsible for rapid incision of ultraviolet irradiated DNA at the site of pyrimidine dimers. Thus, at least three kinds of repair have been postulated to exist in vegetative cells of Dictyostelium discoideum; photoreactivation, excision-repair and post-replication repair. Finally, the existence of yet another repair system, that of a "error-prone" repair pathway had also been proposed for Dictyostelium discoideum by Deering and Sheely (1974). They observed that the excision repair defective Rad C mutant also exhibited enhanced mutability. Hoetzer and Deering in 1980, reexamined the response of Dictyostelium discoideum strain NC4, along with a number of mutants, to UV irradiation in the presence and absence of caffeine. They concluded that the caffeine sensitive repair pathway was possibly "error-

prone". Error-prone repair has been well characterized in E. coli (Witkin, 1976), but evidence for its existence in eukaryotic cells has been marginal (Cleaver, 1978). At any rate, Dictyostelium discoideum vegetative cells appear to have a complex array of repair capabilities which still require further definition.

Thus far in this review, little mention has been made of the repair capacities of spores. The spores of Dictyostelium discoideum are unique in their response to UV irradiation as they are more sensitive than the vegetative cells (Llwerant and Pereira de Silva, 1975; Gillies et al., 1976; Ford and Deering, 1979; Hashimoto and Wada, 1980). This is in marked contrast to the radiation sensitivity of spores of other systems, which are generally more resistant structures than their vegetative counterparts (see Sussman and Halvorson, 1976 for a review). For example, in a study presented by ArShaikely et al., (1971) the UV irradiated spores of Bacillus species exhibit a  $D_{10}$  (10% survival) that is 10 fold higher than the  $D_{10}$  for the vegetative cells. The spores of many bacterial strains are also considerably more resistant than the vegetative cells to ionizing radiation (Tanooka and Sakakibara, 1968). In contrast, though the spores of Dictyostelium discoideum are very UV sensitive, their responses to gamma irradiation are almost the same as that of the vegetative cells (Deering, Smith and Adolf, 1970). This apparently differential response of spores to gamma and UV

irradiation is not unlike that of some of the radiation sensitive (Rad C) mutants which were previously described in this review.

It appears that spores of fungal organisms are also considerably radiation resistant. The growing mycelium in fungal systems is found to be more sensitive than spores (Johnson, 1932). Landen (1939) noticed that the spores of Rhizopus and Ustilago zeae were far more resistant than the sporidia, which in turn were more resistant than yeast or bacterial cells. Some spores, such as those of Aspergillus niger were subjected to fluences as high as  $845,000 \text{ ergs/mm}^2$  ( $84,500 \text{ J/m}^2$ ), to reduce survival to 50 per cent (Zahl, et al., 1939). However, there is an apparent void in the literature concerning the radiation responses of fungal spores, possibly for the reason that they may not be as amenable to radiation research as some other systems. Holleander and Emmons (1939) stated that some of the difficulties in testing the response of fungus spores to radiation included the following: pigmentation (eg. black spores of Rhizopus), clumping, size variation, and the difficulty of assuring that the spores were dead.

Some reviews which have been presented on the subject of the radiation responses of fungal systems include a summary of very early work (Smith, 1936) and also a review by Pomper and Atwood (1955). As it will be observed from the studies presented in this thesis, as well as previous studies discussed, the spores of Dictyostelium discoideum are far more

amenable to radiation research than those of other fungal systems.

A recent study by Ford and Deering (1979) re-examined the UV sensitivity of spores of Dictyostelium discoideum as compared to vegetative cells. They concluded that the additional sensitivity of spores may be due to a UV-induced blockage in a late stage of germination. They do not, however, exclude the possibility that there is also some reduction in repair proficiency during germination and/or early growth on bacteria. Deering found a  $D_{10}$  of  $70 \text{ J/m}^2$  for spores and a  $D_{10}$  of  $160 \text{ J/m}^2$  for vegetative cells (254 nm UV). They found that cells irradiated during development (stationary phase to pre culmination) all had approximately the same sensitivity to UV ( $160 \text{ J/m}^2$ ). Another group of researchers recently reported that the UV sensitivity of cells just after germination was almost equal to that of spores. They found that the sensitivity decreased with development, and reached a minimum just before the first cell division and remained at that level during logarithmic growth. At the stationary phase however, the cells appear to become more sensitive again. This is in agreement with the work of Gillies et al. (1975) who observed that the UV sensitivity of stationary phase cells was comparable to that of the spores. Hashimoto and Wada (1980) postulated that the inferior repair capacity of spores may be due to a lack of repair enzymes or that the enzymes may become functional or be synthesized after germination. Also they speculated that perhaps the radiation




sensitivity of Dictyostelium discoideum is dependent on the cell cycle as was hypothesized for Hela cells (Sinclair, 1969; Downes et al., 1979). Hashimoto and Wada suggest the difference in sensitivity at various stages of the cell cycle may also lead to a difference in sensitivity between spores and amoebae. There is controversy in the literature as to exactly which stage of the cell cycle amoebae are in when they reach the stationary phase. Leach and Ashworth (1972) had suggested that the slower growing axenic amoebae are largely in the  $G_1$  phase of the cell cycle whereas during growth on bacteria, they are in the  $G_2$  phase. Katz and Bourguignon (1974) believe that cells accumulate at a stage late in  $G_2$  just prior to aggregation. Zada-Hames and Ashworth (1978) however, do not believe that all cells are in  $G_2$  at the time of aggregation, and that some cells continue to progress through the cell cycle during the developmental phase. Cells are in the  $G_2$  phase when they have completed DNA synthesis, but not mitotic cell division. Some researchers have shown that there are mitotic cell divisions following aggregation (Bonner and Frascella, 1952) and a "rapid wave" of mitotic divisions just prior to differentiation into spores (Wilson, 1952). Other researchers who agree that Dictyostelium discoideum cells do divide following aggregation, do not believe that this cell division is related to differentiation (Sussman and Sussman, 1960; Loomis, 1971). This is contrary to Zada-Hames and Ashworth (1978) who believe that mitotic cell divisions occurring during development are part of the differentiation program. More recently, Cappuccinelli (1979)

has reported that mitosis was not indispensable, but that it was also not essential for differentiation. In any case, there is still disagreement on the subject of the cell cycle of Dictyostelium discoideum.

Other recent UV work on Dictyostelium has been concerned with the sensitivity of spore formation to UV light. Ohnishi and Nozu (1979) compared the UV sensitivity for killing, i.e. plaque forming ability (PFA), to the UV inactivation of morphogenesis, i.e., fruiting body forming ability (FFA), and found that FFA was 30 times more resistant than PFA. Thus, UV light has less of an effect on morphogenesis than it has on replication. This is reasonable as it is known that DNA synthesis is a more UV sensitive function than RNA or protein synthesis for Dictyostelium discoideum cells (Freim and Deering, 1970). This is because DNA synthesis requires replication of the entire genome; similarly PFA requires cell multiplication which also requires replication of the entire genome, unlike the differentiation process which may not require DNA replication (Ford and Deering, 1979). This may explain the greater resistance of FFA to UV. Ford and Deering (1979) have reported that for repair proficient cells, UV fluences up to  $200 \text{ J/m}^2$  may still allow sufficient transcription for development and differentiation to proceed, whereas, unrepaired lesions in the DNA are sufficient for the blockage of DNA replication (Säuerbier and Hercules, 1978). Indeed, repair proficient NC4 vegetative cells appear capable of completing

development after UV irradiation. Ohnishi and Nozu (1979) found that spores of NC4 were viable even if they were formed after UV irradiation at  $200 \text{ J/m}^2$ , but the  $\gamma$ -sl3 (Rad B mutant) spores were most always inviable. The  $\gamma$ -sl3 mutant was postulated to contain an inheritable unrepaired UV lesion(s) which was responsible for the inviability of the spores formed after UV irradiation (Nozu et al., 1980; Ohnishi et al., 1981). Therefore, it is possible that UV induced lesions can be efficient blocks to macromolecular synthesis in repair deficient organisms such as the  $\gamma$ -sl3 mutant. The fact that spores of NC4 also appear to respond similarly to UV irradiation as some of the repair deficient (Rad C mutant) mutants of Dictyostelium may likewise render the spores particularly suitable for probing macromolecular synthetic activities via UV irradiation.



## MATERIALS AND METHODS

### Production and germination of Dictyostelium discoideum spores

Cultures of Dictyostelium discoideum NC4 (Raper, 1935) were grown on glucose salts agar in conjunction with the bacterium Escherichia coli B/r. The medium was prepared by the addition of the following to 500 ml of distilled water: 6.0 g  $\text{Na}_2\text{HPO}_4$ , 3.0 g  $\text{KH}_2\text{PO}_4$ , 1.0 g  $\text{NH}_4\text{Cl}$  and 0.13 g  $\text{MgSO}_4$ . The salts solution was autoclaved and added to an autoclaved solution consisting of 20 g Difco agar suspended in 500 ml of distilled water. Glucose which had also been sterilized separately was added to the salts-agar mixture immediately after autoclaving. The final concentration was 4.0 g glucose per litre of agar medium. The medium was dispensed to plastic petri dishes and allowed to cool.

Fresh cultures of sorocarps were produced by aseptically transferring a loopful of spores from a stock culture of NC4, to a small volume of sterile distilled water. A loopful of E. coli B/r was also added to this suspension and mixed thoroughly. About 1-2 ml of the mixed spore-E. coli suspension was then aseptically dispensed to the glucose salts agar plates. The plates were incubated at room temperature and shaken periodically to keep the liquid evenly distributed. The plates dried within 2-3 days at which time the aggregating streams of

amoebae had formed migrating slugs. Mature fruiting bodies were allowed to age for an additional 1-3 days prior to harvesting of the spores.

Spores were harvested from the agar plates by passing a moistened glass microscope slide several millimeters above the agar surface, such that it only came in contact with the tops of upright sorocarps. Spores from the sorocarps which adhered to the slide were then suspended in 5- 10 ml of sterile distilled water in a 50 ml beaker. This suspension was transferred to 15 ml test tubes and centrifuged at high speed for about 3 minutes in a Damon/I. E. C. clinical centrifuge. The supernatants from this first wash which contained "crude auto-inhibitor" were saved and pooled with the supernatants from other distilled water washes. The pelleted spores were then resuspended in 5 ml of 10 mM  $\text{PO}_4$  buffer (pH 6.5) and vortexed to eliminate clumping; the spores were re-centrifuged. The washing procedure in fresh  $\text{PO}_4$  buffer was repeated twice for a total of three washings.

Spores to be activated were suspended in a final volume of 5 ml of  $\text{PO}_4$  buffer in a test tube which was placed at 45 C for 30 minutes in a Braun Thermomix II circulating waterbath. After activation, the sides of the tube were carefully wiped to remove any spores that might not have been equally exposed to the heat treatment. Spore concentrations were determined after activation with a hemocytometer, and the densities adjusted with  $\text{PO}_4$  buffer to  $1 \times 10^6$  -  $1 \times 10^7$  spores per ml.

For tube germination, spores were incubated at 23.5 C in

a Braun Thermomix 1420 water bath. The total volume of spore suspension in the 16 x 100 mm tubes did not exceed 5 ml. To assure sufficient oxygenation, the spores were gently stirred with small magnetic stirring bars which were propelled by a submersible stirring unit (Tri-R Corp.).

For large-scale germination experiments, a high density of activated spores was diluted into about 200-300 ml of buffer in 2.8 litre Fernbach flasks such that the spore concentration was again between  $10^6$  -  $10^7$  spores/ml. The flasks were rotated at 150 rpm at room temperature (22-24 C) during germination of the spores.

The percentage of spore swelling and myxamoebae released during the 5 hour germination period was monitored by placing a drop of the cell suspension on a glass slide and counting the first 200 objects under a Zeiss phase microscope at a magnification of 320 x.

#### Isolation and cloning of Dictyostelium discoideum haploid spores

At the time this study was initiated, the spores commonly being used in the laboratory were the diploid strain. Within the diploid population, a small number of haploid spores could always be found. The haploid spores were easily distinguished from the diploid spores by their size and shape when viewed under the phase contrast microscope at 320 x magnification. The diploids were "banana-shaped", while the haploids were oblong, not as curved, and much smaller than the diploids in size (Cotter et al., 1969). Since UV irradiation studies

were to be conducted on Dictyostelium discoideum spores, it was desirable to work with the haploid strain. The haploid spores in the diploid population were selected for and cloned by suspending heat-activated spores serially in tubes of  $\text{PO}_4$  buffer such that the final concentration would yield no more than about 30 spores per 0.1 ml. Aliquots of 0.1 ml of the diluted spore suspension were plated with E. coli and incubated as described for the viability assays; plaques formed on the E. coli lawn about the third day of incubation. Samples of amoebae were scraped only from plaques which were well isolated from each other and were transferred to fresh glucose agar plates. For purposes of economy, the glucose agar plates were subdivided into quarters so that each plate could maintain four randomly chosen clones. Amoebae in each of the clones were able to replicate and eventually differentiate to form sorocarps sufficiently far apart such that there was no interference from neighbouring clones. Samples of spores from sorocarps of each individual clone could be aseptically transferred to a microscope slide for visual screening. Sorocarps which yielded spores which were haploid in appearance were marked, and another sample from the same clone was transferred to one end of an E. coli streak on a fresh glucose salts plate. This method of inoculation allowed the spores to complete the vegetative life cycle with a minimum of migration and thus allowed the stock culture to be kept for a longer period of time (Cotter, unpublished). Spores from such stock cultures could periodically be transferred to fresh agar plates for

sorocarp production as previously described. The working stock cultures were also transferred periodically (about once a month).

#### Ultraviolet light irradiation

For the initial small scale experiments, spores were irradiated at concentrations of  $10^6 - 10^7$  spores per ml in 2 ml volumes of  $\text{PO}_4$  buffer which were placed in open 5 cm diameter glass petri dishes. The dishes were manually swirled during the time of irradiation to assure that all spores received relatively equivalent UV exposure. A General Electric germicidal UV lamp (254 nm) was used for all irradiations usually at a height of 20 cm above the sample. The intensity at a height of 20 cm as measured by a J 225 meter (Ultraviolet Products Inc.) was found to be  $1.7 \text{ J/m}^2/\text{sec}$ . Spores given "fractionated exposures" of UV were irradiated for 5 sec ( $8.5 \text{ J/m}^2$ ) at 15 minute intervals, up until maximum swelling. The intensity of incident radiation was reduced to  $0.5 \text{ J/m}^2/\text{sec}$  for the reciprocity experiments by increasing the height of the UV lamp to 40 cm. The exposure times at the 40 cm height were lengthened to achieve the equivalent total fluences desired. For the radiolabelling experiments, spores were irradiated in 5 ml volumes in 10 cm diameter petri dishes, immediately after activation. For the enzymatic assays, aliquots of spore suspension (10 ml of  $1 - 2 \times 10^7$  spores/ml) were removed from the germination flask and irradiated in 5 ml volumes of the appropriate buffer, in two 10 cm diameter petri dishes. For most



enzyme experiments the two dishes were simultaneously swirled 20 cm below the germicidal lamp for a constant period of 2.5 minutes. The total fluence received by the spores under these conditions was about  $250 \text{ J/m}^2$ . Larger volumes of spore suspension (up to 50 ml) could also be irradiated in 15 cm diameter petri dishes for a period of 2.5 minutes with no detectable occurrence of emergence.

Assay for Viability (plaque forming ability)

Spores were harvested via an alcohol dipped and flamed glass slide into a beaker of sterile distilled water. Washing of spores via centrifugation, and heat activation were performed in sterile test tubes with metal caps. Glass petri dishes (5 cm diameter) were also oven sterilized prior to irradiation. After irradiation the spore suspensions were transferred to sterile test tubes for germination; this test was used as a control to ensure that spores to be assayed for viability would germinate normally after heat activation. The concentration of spores in each tube was determined by hemocytometer counts since variability occurred due to the adhesion of a few spores to the petri dishes. Aliquots of these spore suspensions were then serially diluted (to zero spore concentration) via an Eppendorf pipette (100  $\mu\text{l}$ ) into sterile capped tubes containing 0.9 ml of  $\text{PO}_4$  buffer (Fresh pipette tips never posed any contamination problems, and thus did not require prior sterilization). Aliquots of 0.1 ml or 0.2 ml of the diluted spore suspensions were then spread on dry agar plates

via a sterile glass rod in conjunction with 0.1 ml of a concentrated E. coli suspension. An E. coli lawn formed within 1 - 2 days. Plaques were then counted as they became macroscopically visible on the third and fourth day of incubation at room temperature. Plates were not discarded until after 7 days of incubation in order to detect any late forming plaques as reported by Deering (1979). However by 7 days, fruiting bodies were abundant on the plates and the initial plaques were so large that they ran into one another; accurate estimation of total plaque number was no longer possible after 7 days of incubation. Plates that had not exhibited any plaques by the third and fourth day did not usually exhibit any additional plaques by the end of 7 days. Viability of irradiated spores was determined by the ratio of the plaque counts to the hemocytometer counts. Only plates which yielded significant numbers of plaques (3 - 50) were counted and averaged. The plating efficiency for unirradiated spores averaged about 95 percent.

#### Respiration experiments

Endogenous oxygen uptake was recorded by a YSI model 53 oxygen monitor. Temperature was maintained at 23.5 C in a YSI model 5301 standard bath assembly coupled to a Haake model FE circulator (Cotter et al., 1979). The spore suspensions varied from 3 to 5 ml in volume and spore concentrations were in the range of  $2 - 5 \times 10^6$  spores/ml. Oxygen consumption was recorded as a function of percentage of air saturation at 30 minute

intervals. If the air saturation fell below 55 % during the course of the experiment, the sample was reaerated until levels of over 90 % saturation were again reached. The spores were kept stirring in suspension by small stirring bars (Tri-R Corp.) which were placed inside the sealed chambers. All results were corrected to represent the respiration of spores at a concentration of  $1 \times 10^7$  spores/ml for comparative purposes (Cotter et al., 1979). Cycloheximide addition (200 ug/ml) or UV irradiation ( $250 \text{ J/m}^2$ ) was always performed immediately after heat activation of the spores.

#### Chemical activation and deactivation

For chemically induced activation the spores were suspended as follows: 20 % DMSO for 60 min, 8 M urea for 30 min, 3 M methylene glycol for 60 min, and 30 % methanol for 60 min (Cotter et al., 1976; Cotter and O'Connell, 1976; Cotter, 1977). The spore suspensions were incubated at 23.5 C for the periods indicated, after which they were freed of the chemicals by centrifugation at  $1000 \times g$  for 3 minutes. Pelleted spores were then resuspended in fresh buffer (3 - 4 ml) and germination monitored as previously described. Reversible deactivation was accomplished by suspending spores in 0.25 M sucrose immediately after heat activation (Cotter, 1977). Spores were not washed until the following day. At this time one half of the spore population was reactivated as a control and germination was monitored for 5 hours.

### Radiolabelling experiments

DNA synthesis was evaluated in control spores and UV irradiated spores ( $250 \text{ J/m}^2$ ) by the incorporation of ( $^3\text{H}$ -methyl) thymidine (10 Ci/mmol, ICN) at 5  $\mu\text{Ci/ml}$ , which was added immediately after heat activation. Streptomycin sulfate (Sigma Chem. Co.) was also added to the spore suspensions at a final concentration of 25  $\mu\text{g/ml}$ . Triplicate samples of 0.2 ml were removed every half hour during the 5 hour germination period via a 200  $\mu\text{l}$  Eppendorf micropipette. Each aliquot of spore suspension was transferred into 2 ml of 10 % TCA (0 C) and precipitated material was collected on 24 mm glass fiber filters (Whatman GF/A), and washed with about six 2 ml aliquots of cold 5 % TCA. All filters were dried and their radioactivity determined with a Beckman liquid scintillation counter using Scintisol (Isolab Co.) as a cocktail. The CPMs were corrected for a spore concentration of  $1 \times 10^7$  spores/ml. The non-specific binding of thymidine was not subtracted however, since the counts were very low.

The precursor used to evaluate RNA synthesis was ( $^3\text{H}$ ) uracil (25 Ci/mmol, Amersham Co.) at 5  $\mu\text{Ci/ml}$ . The incorporation into TCA insoluble precipitates of control spores, cycloheximide treated spores (200  $\mu\text{g/ml}$ ) and UV irradiated spores ( $250 \text{ J/m}^2$ ) was performed as described above for thymidine incorporation experiments. In addition to uracil incorporation, the uptake of uracil was also evaluated every half hour, by removing another triplicate set of 0.2 ml aliquots of the same spore suspensions; the suspensions were added to

2 ml volumes of 10 mM  $\text{PO}_4$  buffer, and then the samples were filtered immediately and washed with about six 2 ml volumes of  $\text{PO}_4$  buffer. The first set of samples for uracil incorporation could be held on ice while the phosphate buffer washes were being completed for the uptake samples. The CPMs for both the uracil uptake and incorporation samples were also corrected for a spore concentration of  $1 \times 10^7$  spores/ml and nonspecific binding of uracil was subtracted from all values. While the TCA insoluble counts represented the incorporation into newly synthesized RNA, it was expected that the radioactive counts from the phosphate buffer washes would represent the total accumulation of label into the cells, and thus the values for uptake would include the counts incorporated into the RNA as well as the counts that supposedly accumulated in nucleotide precursor pools.

Protein synthesis was evaluated in control, cycloheximide treated (200 ug/ml) and UV irradiated spores by both the uptake and incorporation of ( $^3\text{H}$ ) leucine (40 Ci/mmol, ICN) at 5 uCi/ml. For incorporation in this case, triplicate samples of 0.2 ml were removed every half hour and transferred into 2 ml of hot 10 % TCA (90 C) for 20 minutes. While this first set of samples was incubated at 90 C, a second set of samples was removed to 2 ml volumes of 10 mM  $\text{PO}_4$  buffer and filtered immediately. This was followed by six 2 ml rinses with  $\text{PO}_4$  buffer containing 1 mM unlabelled leucine, as previously described. The precipitated material of the first set of samples was also collected on glass fibre filters and washed

six times with 2 ml aliquots of cold 5 % TCA containing 1 mM of unlabelled leucine. All discs were dried and counted and the CPMs were corrected for a concentration of  $1 \times 10^7$  spores. The nonspecific binding of leucine was subtracted from all values. The presence of 1 mM unlabelled leucine in the TCA and phosphate buffer washes did not appear to reduce the non-specific binding significantly.

The UV irradiated spores for all the radiolabelling experiments received a fluence of  $250 \text{ J/m}^2$  immediately after heat activation, unless otherwise indicated.

#### Sample preparation for enzymatic assays

For the large scale enzymatic assays, as many as 30 - 40 plates of 2 day old Dictyostelium discoideum haploid spores were harvested and washed as previously described. The spores were heat activated and germinated in 10 mM phosphate buffer (pH 6.5) for the B-glucosidase and trehalase assays, and in 10 mM 2(N-morpholino)ethane sulfonic acid (MES) buffer at pH 6.5 for the alkaline phosphatase assay. Aliquots of germinating spore suspensions (10 ml) were removed at timed intervals, irradiated, and then incubated separately in smaller rotating flasks. The spores and cells were pelleted by low-speed centrifugation and resuspended in 4 or 5 ml of the following buffers: 0.05 M acetate buffer (pH 5.0) for the B-glucosidase assay, 0.1 M acetate buffer (pH 5.5) for the trehalase assay and 0.01 M Tris-HCl, 0.01 M  $\text{MgCl}_2$  (pH 8.5) for the alkaline phosphatase assay. The spores and cells were broken by three

passages through a French pressure cell ( $20,000 \text{ lb/in}^2$ ) and the resulting lysate was frozen immediately in the case of B-glucosidase and trehalase. Frozen samples were thawed just prior to enzymatic assay and all samples were centrifuged at 8000 rpm in a Beckman J21S for 10 minutes to remove cellular debris. The supernatants were removed via pasteur pipettes, placed in clean test tubes, and stored on ice until enzymatic assay. In the case of the alkaline phosphatase enzyme, the lysates after cellular disruption were not frozen, but centrifuged immediately, and then the supernatants were stored on ice until all samples had been disrupted and were ready for assay.

#### Enzymatic assays

The B-glucosidase and alkaline phosphatase assays were performed with slight modifications of previously described methods (Tisa and Cotter, 1979, 1980). The reaction mixtures which contained 0.25 ml of enzyme extract and 0.25 ml of substrate were incubated at 23.5 C. The enzyme and substrate ( $1 \times 10^{-2} \text{ M}$  p-nitrophenyl B-D-glucoside, Sigma Chem. Co.) for the B-glucosidase assay were suspended in 0.05 M acetate buffer pH 5.0, and incubated for 50 minutes. The enzyme and substrate ( $1 \times 10^{-2} \text{ M}$  p-nitrophenyl phosphate, Sigma Chem. Co.) for the alkaline phosphatase determination were suspended in buffer (0.01 M Tris-HCl, 0.01 M  $\text{MgCl}_2$ , pH 8.5) and incubated for 20 or 30 minutes. All reactions were stopped by the addition of 1 ml of 1 M  $\text{Na}_2\text{CO}_3$  at the appropriate times. The optical.

density at 420 nm was measured with a Spectro-plus single beam spectrophotometer. In all cases the measurements were corrected for spontaneous substrate hydrolysis. It should be mentioned that there is considerable hydrolysis of substrate which occurs with time after the addition of  $\text{Na}_2\text{CO}_3$ . Therefore, samples must be read immediately after the addition of  $\text{Na}_2\text{CO}_3$ . When assaying a large number of samples taken throughout the germination period as is done in these experiments, it is possible to set up a standard curve which accounts for "substrate hydrolysis with time" after the addition of carbonate. The degradation appears to follow linear kinetics within at least the first hour of readings (data not shown). The time of reading of enzyme samples can be recorded and the proper substrate corrections made. For this reason it is also desirable for the blank to contain only the appropriate buffer and the added carbonate, so that it will not change in optical density with time during the reading of a large number of samples. A unit of activity was defined as that amount of enzyme which released 1 nmole of p-nitrophenyl per minute under these conditions. The specific activity is expressed as units of enzyme activity per milligram of released protein.

Trehalase activity was assayed by adding 1 ml of spore extract containing 50 - 200 ug of protein to 1 ml of trehalose (50 mM stock) dissolved in 0.1 M acetate buffer pH 5.5. The reaction mixture of 2 ml was incubated at 35 C for 30 minutes and then the reaction was terminated by boiling for 10 minutes in loosely capped test tubes. Zero time samples were boiled



for 2 minutes prior to the addition of substrate and for an additional 10 minutes immediately after the substrate had been added. A substrate control was included to determine spontaneous degradation of substrate. The precipitate that formed during the boiling step was pelleted by low speed centrifugation. Aliquots of 0.5 ml or 1.0 ml of the reaction mixture containing glucose were removed to fresh tubes to which an equal volume of glucose reagent was added. The glucose reagent contained 45 U/ml of glucose oxidase (Miles Biochem. Co.), 7.5 U/ml of horseradish peroxidase (Miles Biochem. Co.), and 2.3  $\mu$ moles/ml of 2,2'-Azino-di-(3-ethyl-benzthiazolin)-6-sulfonate (Boehringer Mannheim) in 0.1 M acetate buffer at pH 5.5 (Chan and Cotter, in press). The optical density at 420 nm was determined after 45 minutes of incubation at 23.5 C and  $\mu$ moles of glucose could be obtained from a standard curve with glucose. One unit of enzyme activity was defined as that amount of enzyme which released 1  $\mu$ mole of glucose per 30 minutes under the above conditions. (The units for enzyme activity could also be converted from  $\mu$ moles/30 min to nmoles/min by multiplying by a factor of 33.33). The specific activity was again expressed as units of enzyme activity per milligram or released protein.

#### Protein determination

Protein was measured by the method of Bradford (1976) using albumin as a standard. The albumin was dissolved in each of the appropriate buffers for the respective enzymes

that were used in the extraction procedures. It should be mentioned that the use of albumin as a protein standard with the Bradford reagent results in protein readings which yield specific activity values that are approximately twice the values obtained when gamma globulin is used for the protein standard curve. All values for specific activity are relative to each other, provided the same standard for protein is used. Thus if it is necessary, it is possible to divide the specific activities obtained with albumin as a standard by 2, in order to compare the experimental values with those observed in the literature.

## RESULTS

### Part I Physiological Effects of Ultraviolet Light on Spore Germination of Dictyostelium discoideum

#### 1. Emergence of myxamoebae from irradiated spores

The inactivation kinetics for spores irradiated immediately after activation are presented in Fig. 2. With increasing UV fluence ( $8.5\text{--}50\text{ J/m}^2$ ) a greater percentage of the population of spores was inhibited from releasing amoebae. Though there was variation between independent experiments, as indicated by the vertical bars which show the range of values, the percentage emerged at 24 hours was consistently higher than that observed 5 hours after UV treatment. A UV fluence of about  $50\text{ J/m}^2$  (30 sec of UV exposure) was sufficient to reduce emergence to less than 1% of control values when measured 5 hours after treatment; the same fluence allowed an average of about 6% emergence when measured after 24 hours. When a total fluence of  $77\text{ J/m}^2$  (45 sec of UV exposure) was given, less than 1% emergence occurred at either 5 or 24 hours after UV treatment (Fig. 2).

Organisms which have effective excision repair mechanisms are known to exhibit greater survival after fractionated irradiation than after equivalent fluences applied in a single UV exposure (Harm, 1968; Ferguson and Cox, 1979). The percent emergence of myxamoebae from spores which received

Figure 2.

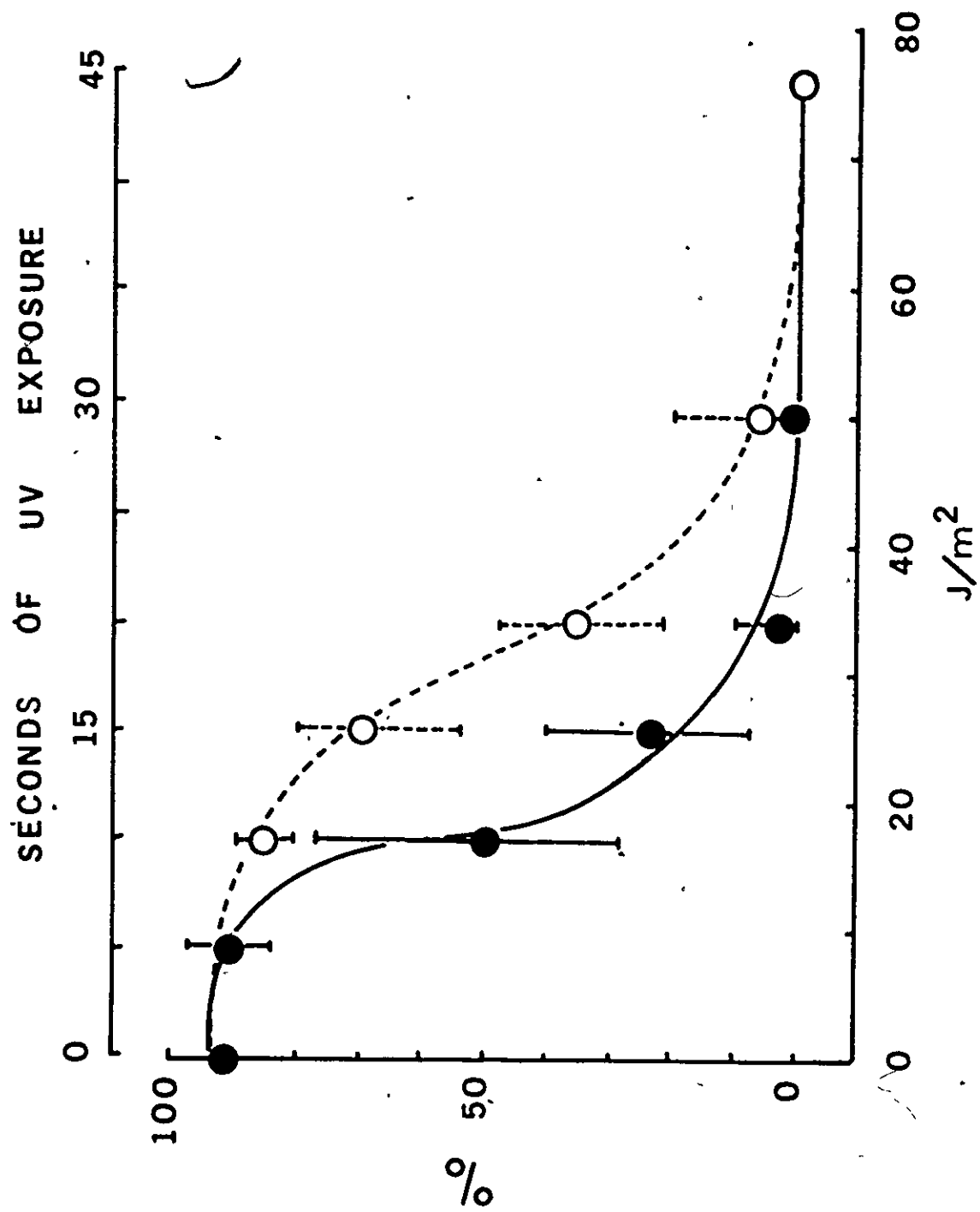
The percent emergence of amoebae from activated spores irradiated with a fluence of  $1.7 \text{ J/m}^2/\text{sec}$ . Activated spores were irradiated for various times and then incubated for 5 or 24 hours at 23.5 C. The percent myxamoebae emergence is plotted versus both the time of exposure and the total fluence.

Symbols: ( ● ) percent emergence 5 hours after activation  
(data is averaged from 10 independent experiments)

( ○ ) percent emergence 24 hours after activation  
(data is averaged from the 7 experiments that were continued for 24 hours)

The vertical dashed and solid lines indicate the range of values obtained in the experiments.

Note: see Table 1 for a comparison of total UV fluences applied at different fluence rates for various exposure times.



"fractionated exposures" of UV is plotted in Fig. 3. The vertical lines of Fig. 2 which indicated the range of values for final percent emergence for spores receiving single UV exposures have been superimposed on the data of Fig. 3 for the sake of comparison. There is no enhancement of the final percent emergence of spores given cumulative "fractionated exposures" of UV when compared to spores that received equivalent total single UV exposures. Spores given cumulative fractionated exposures of UV which totalled a fluence of  $77 \text{ J/m}^2$  (45 sec of UV exposure) also produced less than 1% emergence of myxamoebae after both 5 hours or 24 hours of incubation (Fig. 2).

In another experiment spores were exposed to a lower fluence rate of UV irradiation ( $0.5 \text{ J/m}^2/\text{sec}$ ) for longer exposure times such that they received equivalent total UV fluences (Fig. 4). It has been postulated that spores should show a higher efficiency of emergence at a low UV fluence rate, as opposed to a high UV fluence rate, if they have efficient dark repair mechanisms (Owens and Krizek, 1980). Such spores do not exhibit reciprocity. From the data of Fig. 3 it appears that Dictyostelium discoideum spores did not exhibit any enhanced emergence at the lower fluence rate. The final percent emergence at 5 hours and 24 hours for spores irradiated at the low fluence rate ( $0.5 \text{ J/m}^2/\text{sec}$ ) were comparable to the range of values observed for spores irradiated at the higher fluence rate ( $1.7 \text{ J/m}^2/\text{sec}$ ). Again the vertical ranges of Fig. 2 have been superimposed on the values of Fig. 4

Figure 3.

The percent emergence of amoebae from activated spores receiving fractionated exposures to UV. Spores were irradiated with  $1.7 \text{ J/m}^2/\text{sec}$  for 5 sec exposures at 15 minute intervals up until maximum swelling. Spores thus received a cumulative fractionated exposure of UV which corresponded to the total fluence received by spores given a single UV exposure

Symbols: ( ■ ) percent emergence of myxamoebae 5 hours after activation (fractionated exposure)  
 ( □ · ) percent emergence of myxamoebae 24 hours after activation (fractionated exposure)

The data shown was obtained from 1 typical fractionated exposure experiment. For comparative purposes, the vertical solid and dashed lines indicate the range of emergence values for spores receiving single UV exposures (replotted from Fig. 2).

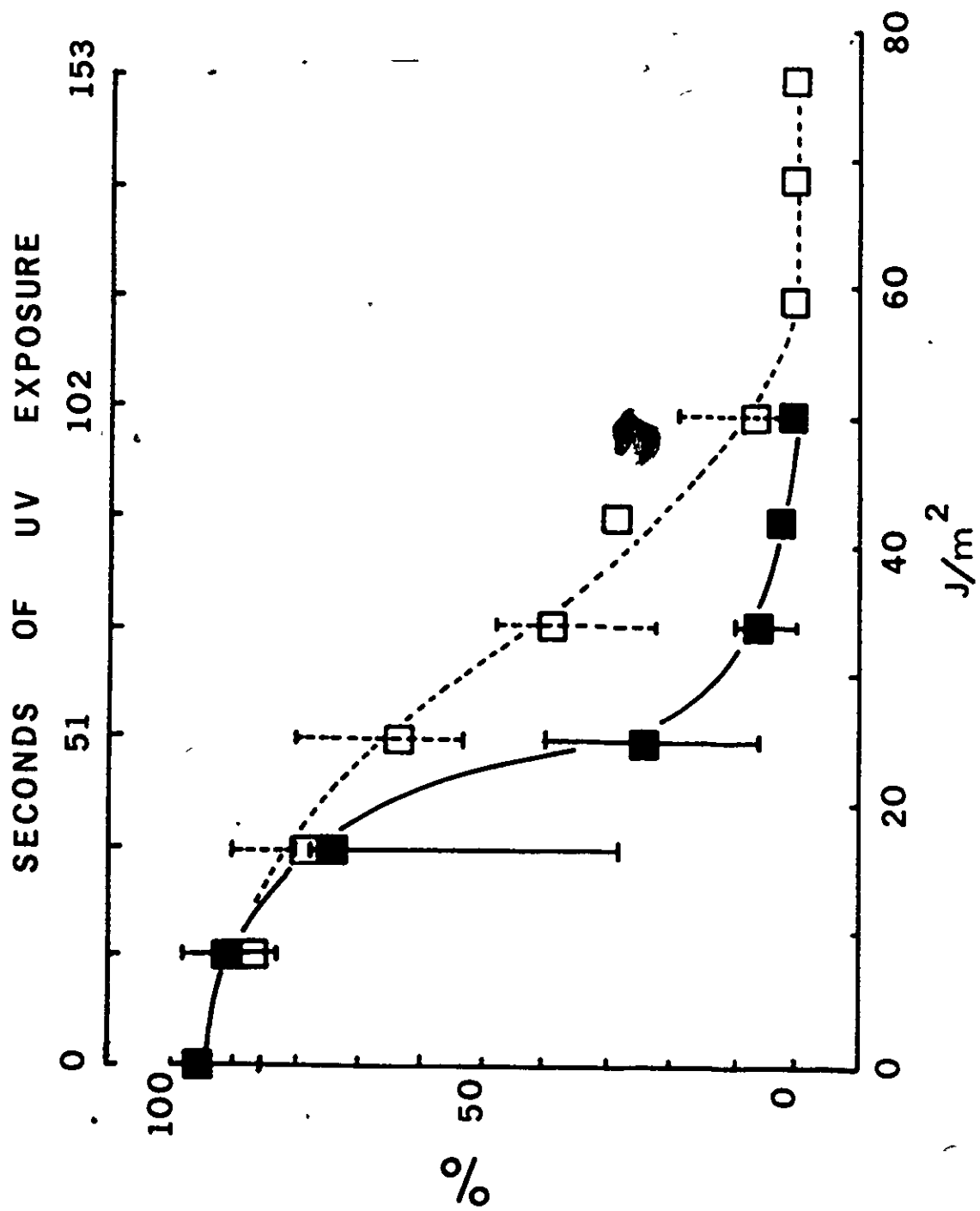


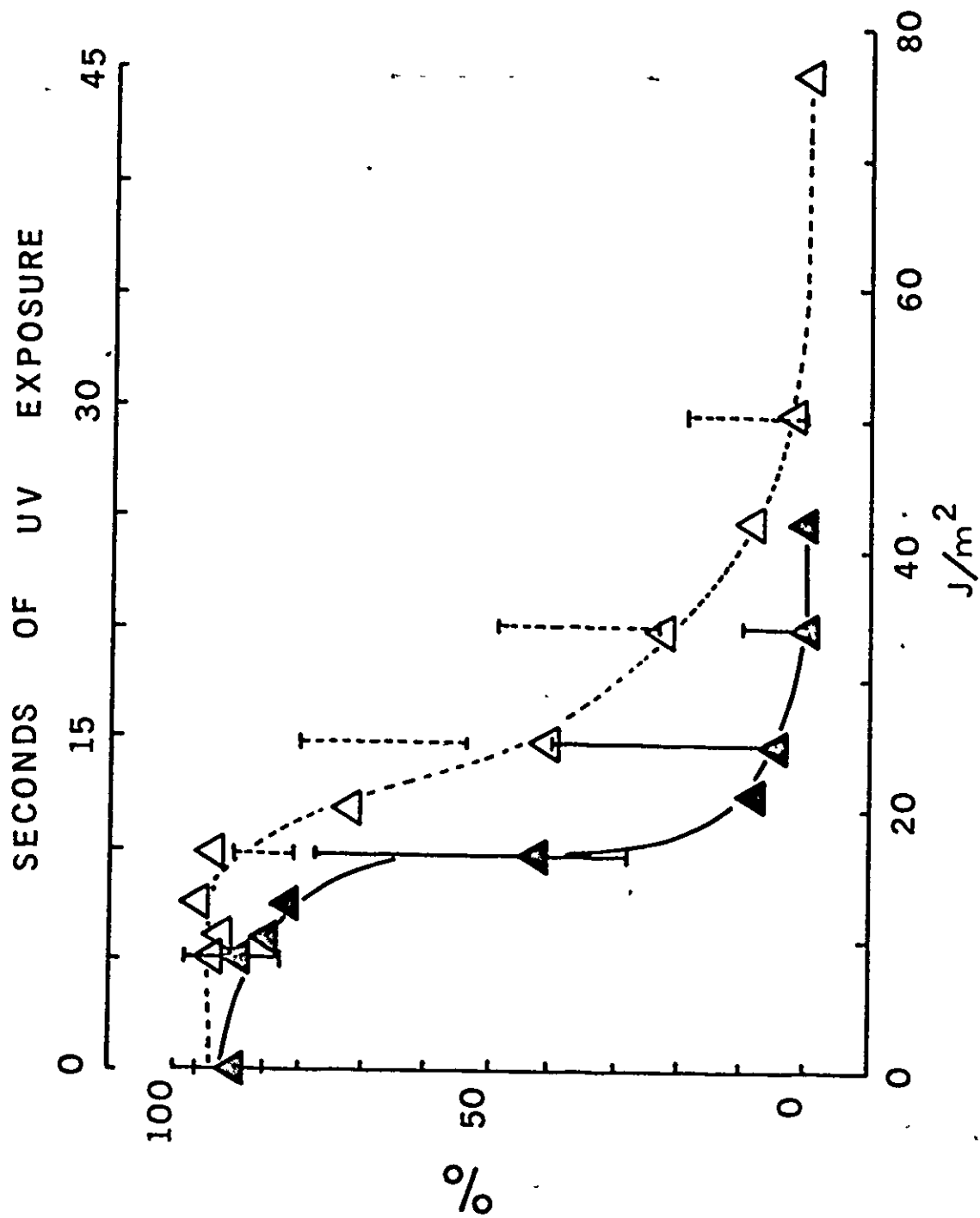


Figure 4.

The percent emergence of amoebae from activated spores irradiated with  $0.5 \text{ J/m}^2/\text{sec}$ . Spores were exposed to the lower UV fluence for longer time periods such that they received a total UV fluence which corresponded to the total fluence received by spores irradiated at a fluence rate of  $1.7 \text{ J/m}^2/\text{sec}$  (see Fig. 2).

Symbols: ( ▲ ) percent emergence of myxamoebae 5 hours after activation  
 ( △ ) percent emergence of myxamoebae 24 hours after activation

The data shown is from 1 typical experiment. For comparative purposes, the vertical solid and dashed lines indicate the range of emergence values obtained for spores irradiated with UV at a fluence rate of  $1.7 \text{ J/m}^2/\text{sec}$  (replotted from Fig. 2).



for the sake of comparison. The values in Fig. 3 do, however, appear to be in the lower ranges of percent emergence expected for the equivalent total fluence. It is possible that the longer UV exposure times, even at the lower fluence rate (17 sec as opposed to 5 sec) allow the UV light to reach more of the spores. Thus equivalent total fluences at the lower UV rate may only appear to be more inhibitory. A comparison of the total fluences applied at various fluence rates for various exposure times is shown in Table 1.

In a number of the experiments which were used to determine the average percent emergence of UV irradiated haploid spores in Fig. 2, the effect of UV irradiation upon diploid spores was also monitored. Diploid spores are easily distinguished from the haploid spores on the basis of their larger size and their characteristic "banana" shape (Cotter et al., 1969). A similar range of values for percent emergence at various total fluences is observed for both the diploid and haploid spores (Table 2). It was thought that the diploid spores, which have been shown to have twice the DNA content of the haploid spores (Cotter, Phd. Thesis) might exhibit greater UV resistance than the haploid spores. The diploid resistance should be reflected in higher values for the average emergence after UV irradiation. However, the explanation of the UV response of haploid and diploid spores is more complex than that proposed in basic target theory. It has been shown that the sensitivity of diploid amoebae of Dictyostelium discoideum which were constructed by the fusion

Table 1

Summary of UV Fluences Used with Comparison of Units and  
Exposure Times at Various Fluence Rates

UV Total Fluence		Exposure Time (seconds)	
ergs/mm. <sup>2</sup>	J/m <sup>2</sup>	20 cm Distance <sup>a</sup>	40 cm Distance <sup>b</sup>
85	8.5	5	17
100	10.0	-	20
125	12.5	-	25
170	17.0	10	34
215	21.5	-	43
255	25.5	15	51
340	34.0	20	68
425	42.5	25	85
510	51.0	30	102
595	59.5	35	119
680	68.0	40	136
765	76.5	45	153
1,020	102.0	60	-
1,530	153.0	90	-
2,040	204.0	120	-
2,550	255.0	150 <sup>c</sup>	-
5,100	510.0	300	-

a The fluence rate at a lamp height of 20 cm was found to be 1.7 J/m<sup>2</sup>/sec

b The fluence rate at a lamp height of 40 cm was found to be about 0.5 J/m<sup>2</sup>

c This is the total exposure time which is used in the majority of the experiments reported in the course of this thesis.

Table 2

Percent Emergence of Myxamoebae from Haploid versus Diploid  
Spores after UV Irradiation

UV Exposure Time (seconds)	Percent Emergence from Haploid Spores		Percent Emergence from Diploid Spores	
	Range of Values	Average Value	Range of Values	Average Values
0	86.5 - 99.0	93.0	89.5 - 98.0	93.3
5	84.0 - 94.0	92.9	89.0 - 98.0	93.9
10	28.0 - 84.0	57.2	50.0 - 80.5	63.9
15	6.5 - 62.0	24.1	16.0 - 63.5	29.8
20	0 - 9.5	5.3	1.5 - 25.5	8.4
30	0 - 0.5	0.1	0 - 2.5	0.7
45	0 - 0	0	0 - 0	0

The data presented are the average of 6 independent experiments. Haploid and Diploid spores were irradiated for various times immediately after activation at a fluence rate of  $1.7 \text{ J/m}^2/\text{sec}$ . The percent of emerged myxamoebae was determined 5 hours after heat activation.

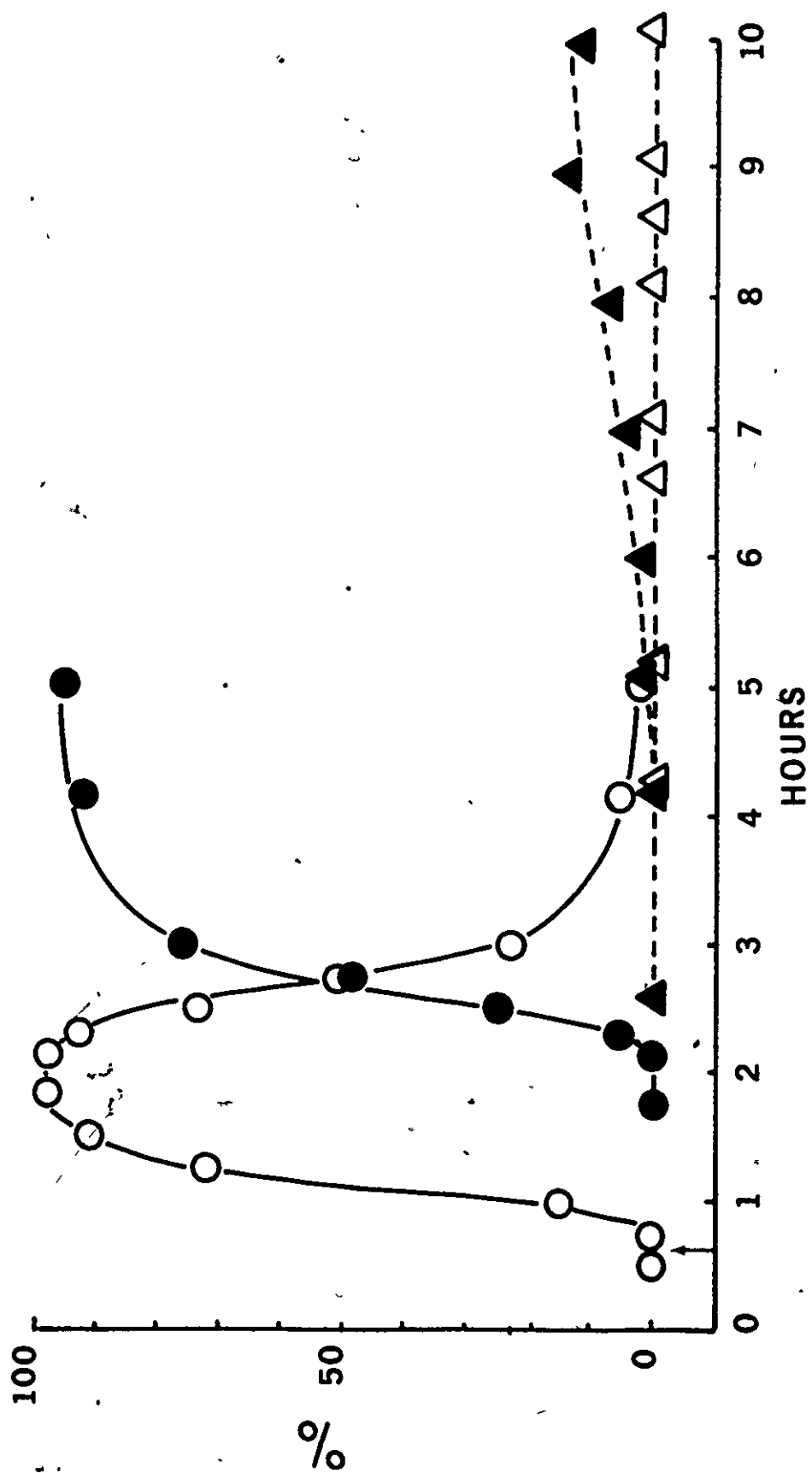
of two radiation resistant haploids exhibited relatively the same survival as the haploid wildtype (NC4) amoebae (Welker and Deering, 1978). The fact that the UV sensitivity of cells is not entirely reflected in the number of genome equivalents per cell, has been observed in other systems as well (Calkins, 1968).

The kinetics for emergence of myxamoebae from unirradiated spores is shown in Fig. 5. It has been demonstrated in Fig. 2, Fig. 3 and Fig. 4 that there is an increase in the percent of emerged myxamoebae between 5 and 24 hours after activation when spores are irradiated with UV fluences between 20 and 50 J/m<sup>2</sup>. Thus, at "sublethal" UV fluences, a small number of spores is still capable of releasing amoebae though the process is somewhat delayed from that of unirradiated spores (Fig. 5). In the case for the data plotted in Fig. 5, spores were irradiated with a fluence of 50 J/m<sup>2</sup> (30 sec UV exposure) immediately after activation, and then transferred to glucose salts plates where the emergence was monitored for ten hours. Spores irradiated with about 200 J/m<sup>2</sup> (120 sec UV exposure) were also monitored as a control for complete emergence inhibition. A very low percent of emergence could first be detected in the irradiated spores (50 J/m<sup>2</sup>) at 5 hours, and this value only increased to about 10-15% by 10 hours. This is in good agreement with values observed in previously described experiments. Such delays are not uncommon in many organisms that are irradiated with sublethal UV fluences. The application of UV is known to cause delays in

Figure 5.

The delay of myxamoebae emergence from spores irradiated with sublethal UV fluences. Heat activated spores were irradiated at a fluence rate of  $1.7 \text{ J/m}^2/\text{sec}$  immediately after activation as indicated by the arrow. The data presented is from 1 independent experiment.

Symbols: ( ○ ) percent swelling of unirradiated spores  
( ● ) percent emergence of unirradiated spores  
( ▲ ) percent emergence of myxamoebae from spores exposed to 30 sec of UV light  
( △ ) percent emergence of myxamoebae from spores exposed to 120 sec of UV light





the cellular synthesis of macromolecules which in turn have varied consequences such as cell division delay and growth delay (Mitchison, 1971; Harm, 1980). The delay in emergence of spores irradiated with sublethal fluences may be due to some minimal capacity that the spores may have in recovering from sublethal UV damage. It should be noted that no recovery is apparent after 45 sec UV exposure times, and thus no delayed emergence is observed once the total UV fluence is this high.

## 2. Viability of irradiated spores

The viability of irradiated spores as measured by plaque forming ability can be seen in Fig. 6. With increasing UV fluence ( $25-100 \text{ J/m}^2$ ) the survival decreased logarithmically after a slight shoulder. Survival curves of repair deficient organisms characteristically exhibit slight shoulders (Harm, 1980). The viability after a fluence of  $\approx 50 \text{ J/m}^2$  was reduced to an average value of about 10%. This is in fair agreement with the emergence data of Fig. 2 and suggests that the number of myxamoebae observed by 24 hours after UV may represent the fraction of the population that is capable of replicating to produce plaques. UV fluences in excess of  $100 \text{ J/m}^2$  (60 sec UV exposure) reduce survival to below 0.1%. At these higher fluences, the slope of the curve appears to deviate considerably. This levelling off of the slope may be indicative of the existence of a small number of resistant spores in the population; however, the apparent resistance of some cells in large populations may also arise due to chance. It is known





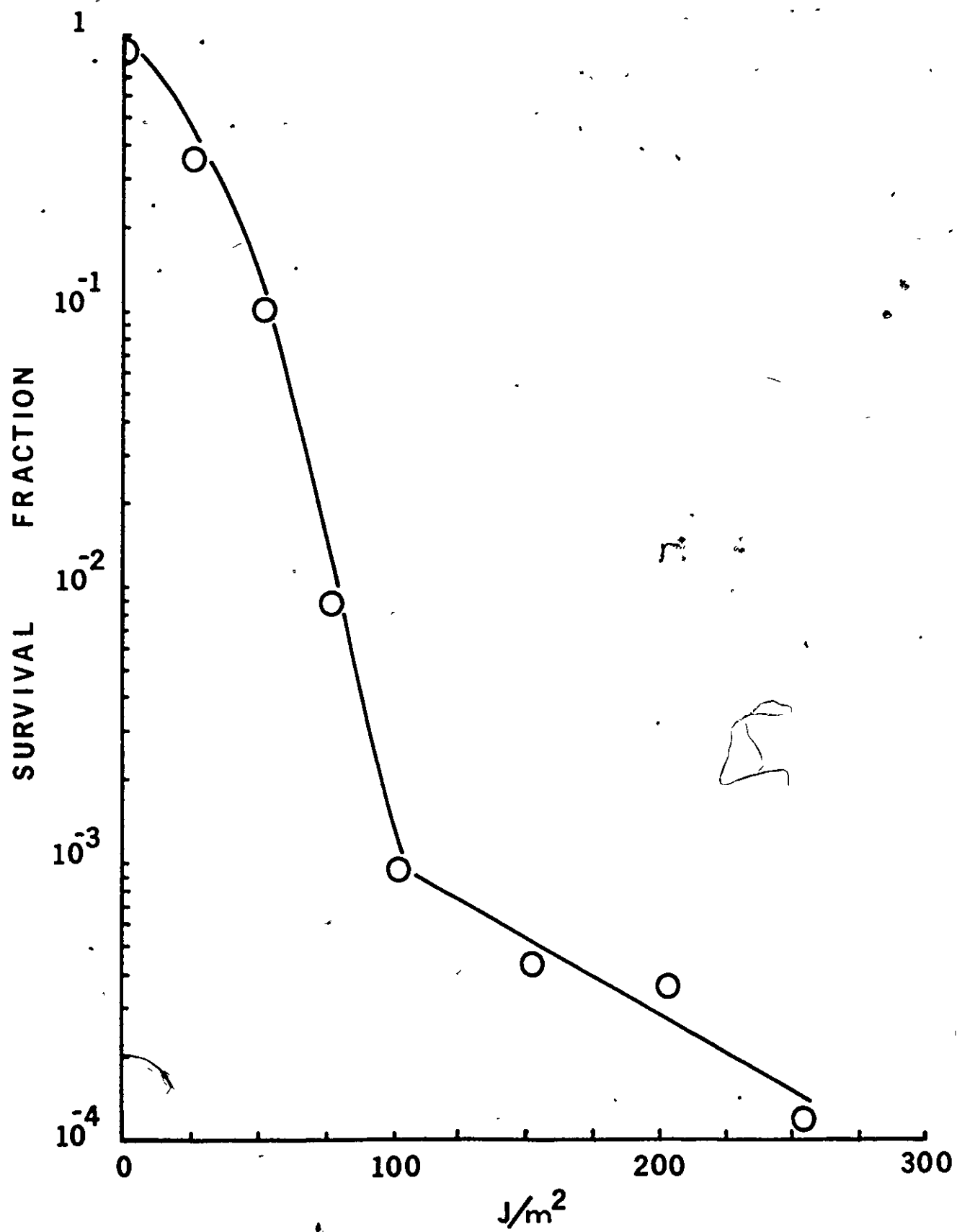


Figure 6.

Viability of activated spores irradiated with  $1.7 \text{ J/m}^2/\text{sec}$ .

Spores were activated and irradiated for various times before plating. The viability was determined according to the procedure outlined in the Materials and Methods section.

Symbol: (  ) average survival fraction as determined from 3 independent experiments.



that only a certain number of hits can be made from a given amount of irradiation, and thus the cells which survive may be the ones not hit. It has been argued that it is unrealistic to quote the fluence required to kill all cells, since usually a few will escape any amount of radiation (Hilyard and Biggin, 1977).

### 3. Irradiation during spore germination

UV irradiation of spores at any time prior to late spore swelling allows full swelling but completely prevents the emergence of myxamoebae (Fig. 7). The slightly slower swelling observed for spores irradiated prior to or immediately after heat activation does not appear significant since the majority of spores reach maximum swelling at about the same time as unirradiated controls. The arrows indicate the time of application of a fluence of  $250 \text{ J/m}^2$  to 2 ml aliquots of a spore suspension which were removed from the flask of germinating spores. It is implied that irradiation at any time interval in between the first and last arrow of Fig. 7 is completely inhibitory to emergence. The percent emergence for these aliquots was scored in separate test tubes.

UV irradiation of spores at any time after late spore swelling allows emergence to occur in a certain fraction of the population. The kinetics of this emergence can be seen in Fig. 8, which are the data from one typical experiment. Spores were irradiated at a time, as indicated by the arrow, when emergence had already begun in the population. It is noted

Figure 7.

The effect of UV upon spores irradiated prior to late spore swelling. Arrows indicate the time of irradiation ( $250 \text{ J/m}^2$ ) in three separate experiments.

- Symbols: (■) time of heat activation
- (○) percent swelling of control spores
  - (●) percent emergence of myxamoebae from control spores
  - (△) percent swelling of spores irradiated 15 min prior to activation
  - (▲) percent emergence of myxamoebae from spores irradiated 15 min prior to activation
  - (□) percent swelling of spores irradiated 6 min after activation
  - (■) percent emergence of myxamoebae from spores irradiated 6 min after activation
  - (⬡) percent swelling of spores irradiated 2 1/4 hr after activation
  - (⬢) percent emergence of myxamoebae from spores irradiated 2 1/4 hr after activation

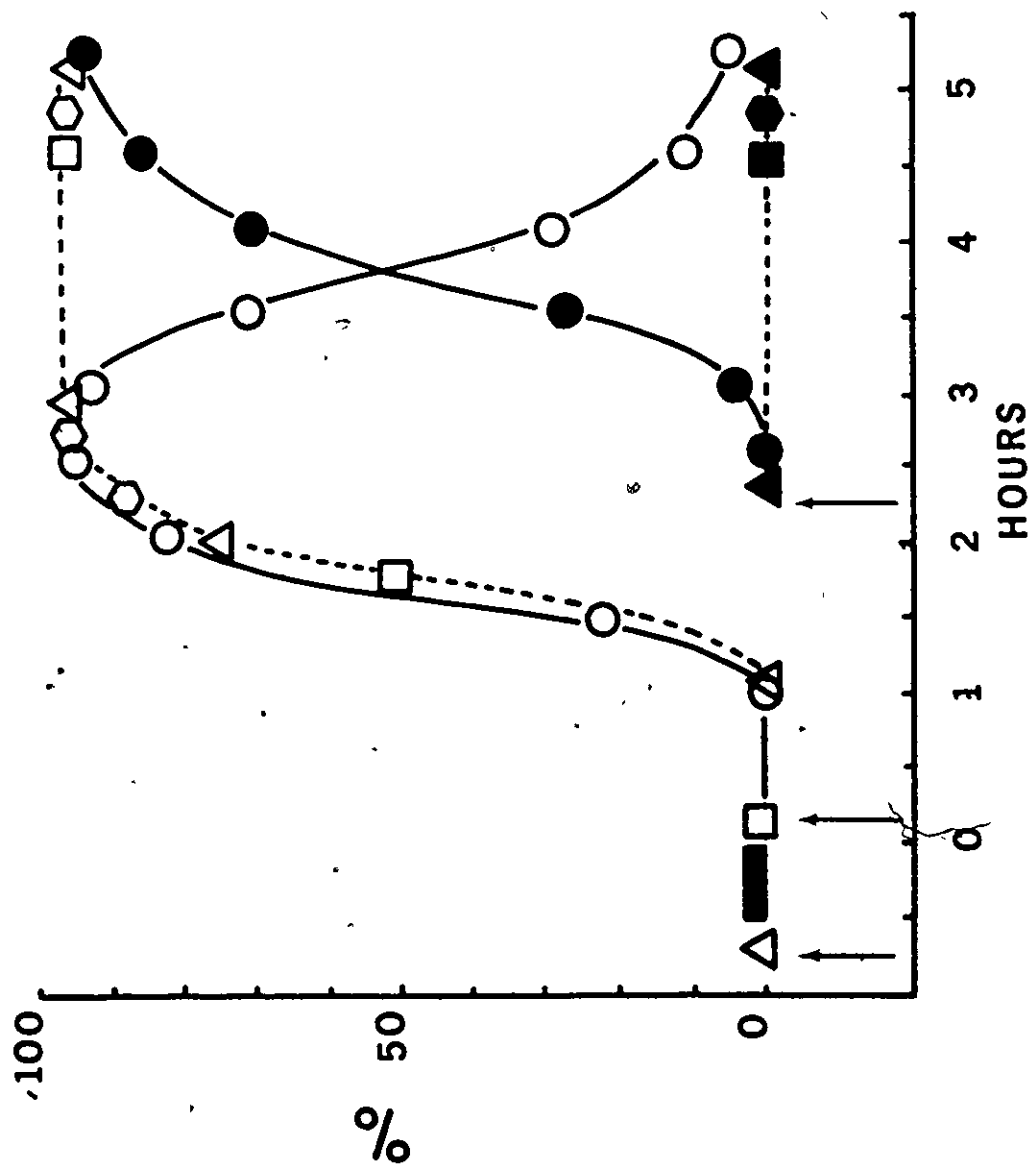
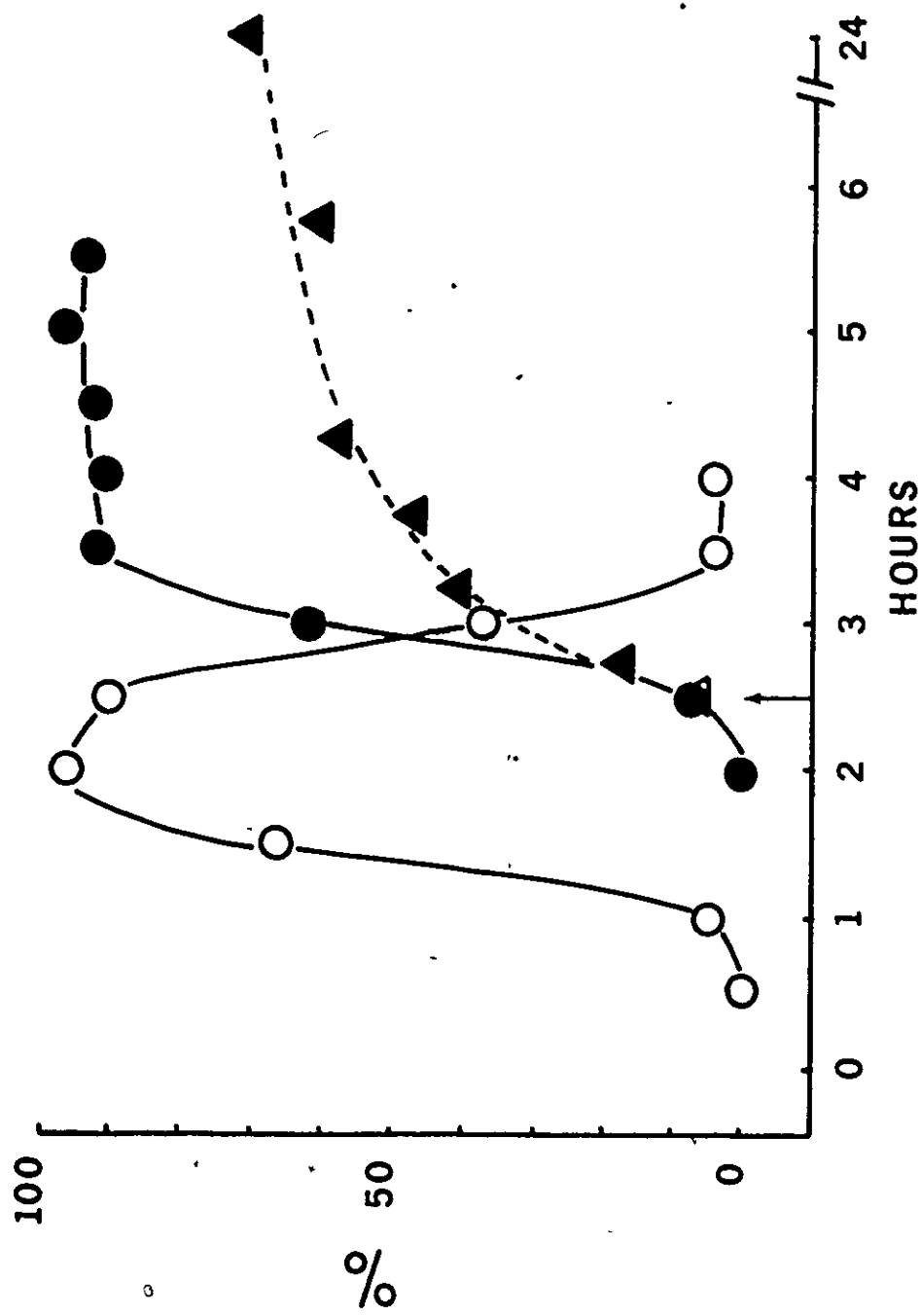


Figure 8.

Emergence kinetics of activated spores irradiated after late spore swelling. The arrow indicates the time of irradiation ( $250 \text{ J/m}^2$ ) in 1 independent experiment.

Symbols: ( ○ ) percent swelling of control spores  
( ● ) percent emergence of myxamoebae from control spores  
( △ ) percent emergence at the time of UV irradiation  
( ▲ ) percent emergence following UV irradiation





that emergence is not immediately inhibited at the time of UV application, and in fact, continues for some time, and then gradually levels off. The final percent emergence by 24 hours may be only slightly higher than that observed by 6 hours after activation. It cannot be excluded that some of the amoebae observed by 24 hours are due to cell division.

In Fig. 9a, UV was applied at a number of intervals just before and during the emergence of myxamoebae. It is again observed that the UV irradiation of spores after late spore swelling, allows emergence to occur in only a certain fraction of the population. This initially small fraction is further along in the germination sequence than the majority, and is no longer inhibited from emerging by UV light. It may not be quite apparent from Fig. 9a, which indicates 10 different exposure times, but the application of UV at  $2\frac{1}{2}$  and  $2\frac{3}{4}$  hours after activation (still no amoebae evident in the population at the time of irradiation) resulted in 1% and 8% emergence respectively by 6 hours. Similarly, when UV was applied 3 hours and later after activation, there was some additional emergence of myxamoebae following irradiation (Fig. 9a). In Fig. 9b, a portion of the data of Fig. 9a is replotted such that the final percent emergence (at 6 hours) is shown at the time of UV application (The 6 hour values are plotted above the arrows indicating the respective times of UV application). The values of percent emergence attained by irradiated spores 6 hours after activation follows a sigmoidal distribution, which is relatively parallel to the values of percent emergence at the time of UV application.

Figure 9a.

The effect of UV upon activated spores irradiated after late spore swelling. Arrows indicate the time of irradiation ( $250 \text{ J/m}^2$ ) in ten separate experiments.

- Symbols:
- ( ○ ) percent swelling of control spores
  - ( ● ) percent emergence of myxamoebae from control spores
  - ( △ ) percent emergence at the time of UV irradiation
  - ( ▲ ) percent emergence of myxamoebae from spores following UV irradiation

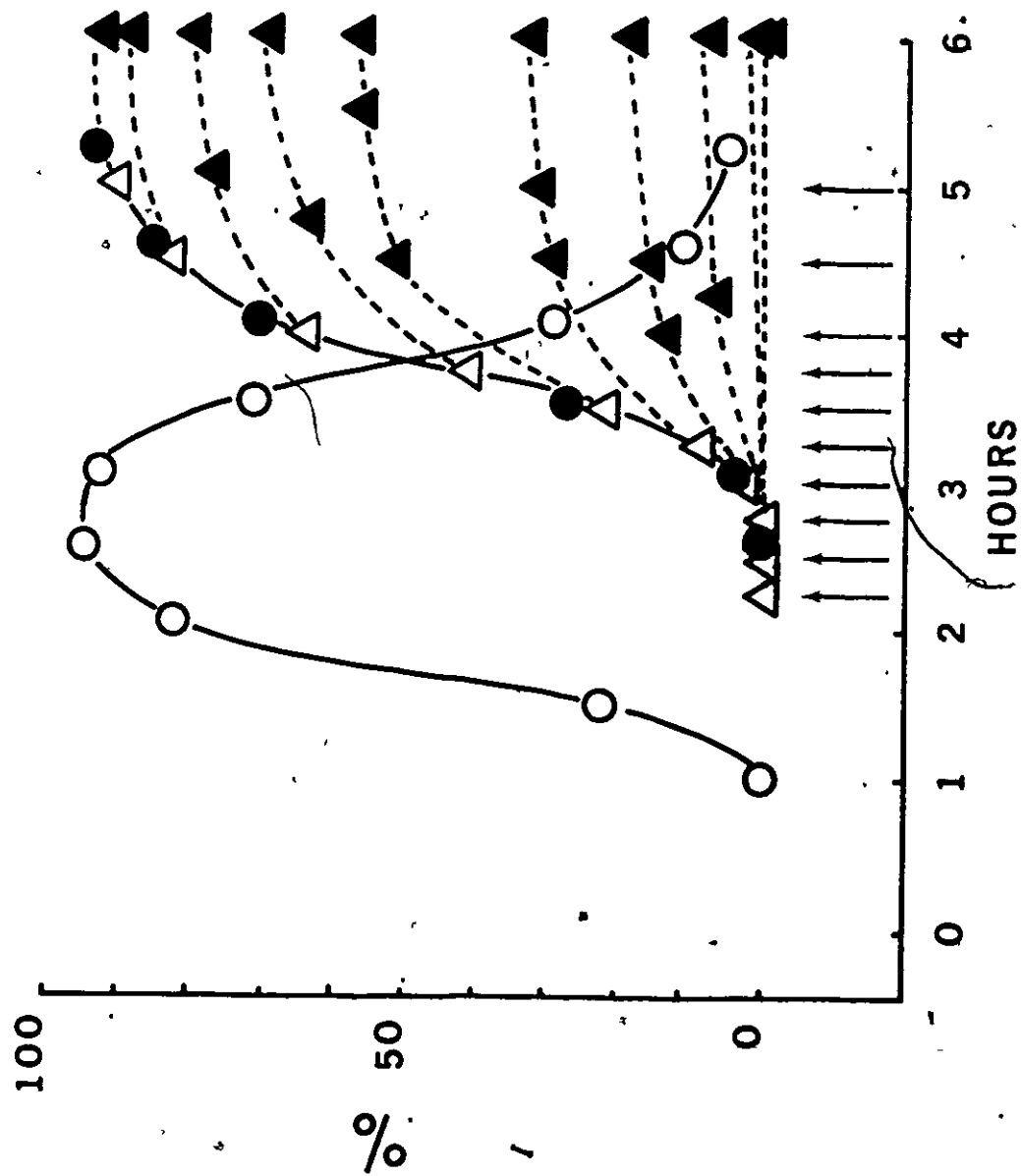
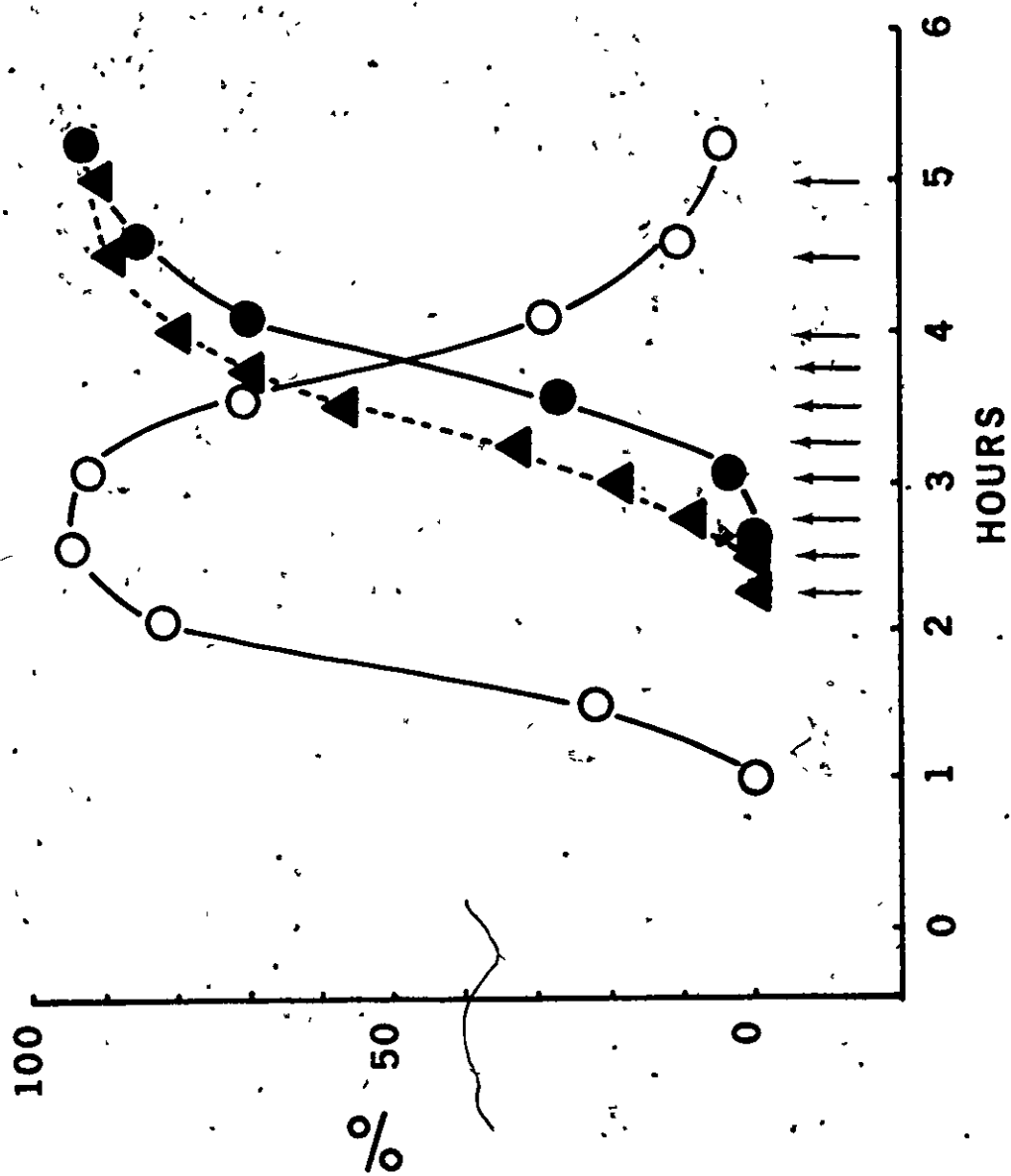


Figure 9b.

The final percent emergence of myxamoebae from spores irradiated after late spore swelling. The arrows indicate the time of irradiation ( $250 \text{ J/m}^2$ ) of activated spores, in ten separate experiments. The final percent emergence 6 hours after activation is plotted against the time of UV application.

- Symbols: ( ○ ) percent swelling of control spores  
( ● ) percent emergence of myxamoebae from control spores  
( ▲ ) final percent emergence of myxamoebae from irradiated spores 6 hours after activation





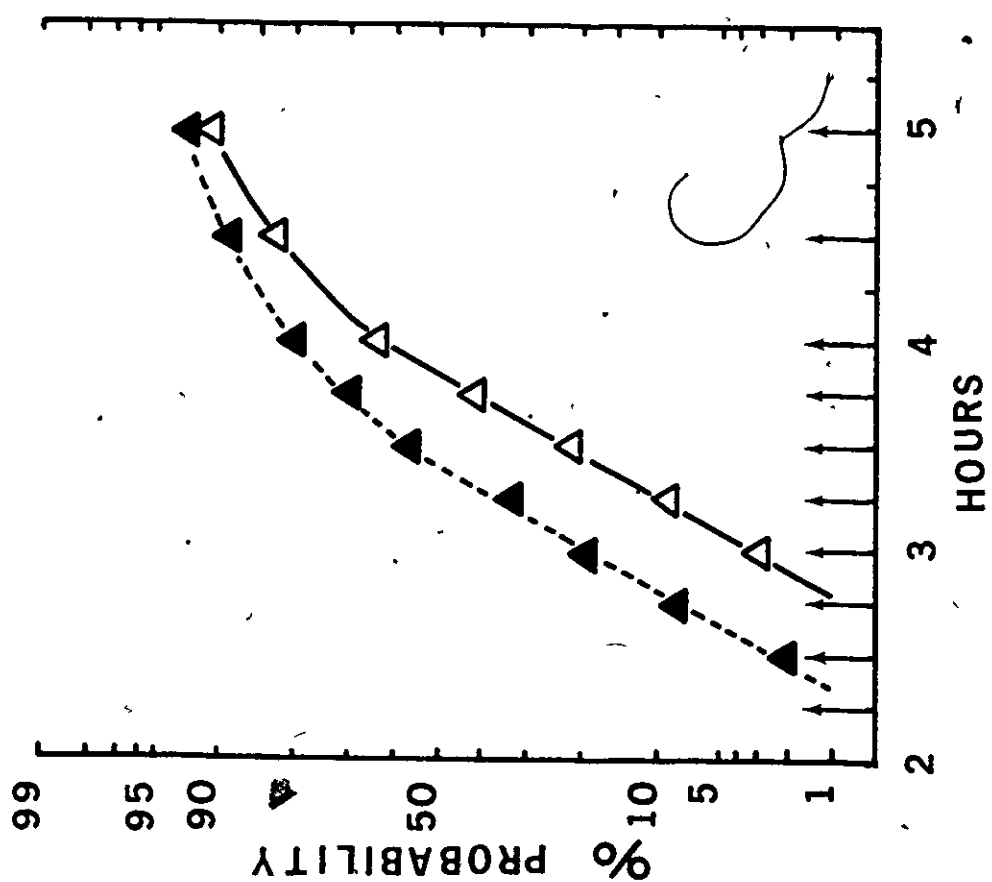
It is apparent from the data of Fig. 9a and 9b that at some time, just prior to emergence, UV is no longer inhibitory to that fraction of the population which has reached this point in the germination sequence. We have defined this point as the "competence point". The data of Fig. 9b can also be re-plotted on a probability scale. The plotting of a sigmoidal germination curve on a probability scale will result in a straight line; any deviations from a straight line indicate that the data deviate from a sigmoidal function. The probability plot is most useful when used to compare sigmoidal functions which may bare temporal relationships. Such curves should be parallel when the data is plotted on a probability scale. The temporal distance between the two curves in Fig. 9c (at 10% probability) was used to estimate the competence point; the minimal estimate from Fig. 8 is that competence is reached approximately 27-30 minutes prior to emergence. It is a minimal estimate since a few additional myxamoebae may emerge in the irradiated population later than 6 hours after activation. The data of Fig. 9c also demonstrate that emergence curves deviate from a sigmoidal function at the higher germination percentages; this is universally found for germination curves of microorganisms (Sussman and Halvorson, 1966).

It should be noted that competence can be considered an all or none response for any individual amoebae in the population. The amoebae will or will not emerge from the spore. The all or none response for emergence is evident at the 2.5 minute (150 sec) UV exposure used to determine the competence

Figure 9c.

The final percent emergence of myxamoebae from spores irradiated after late spore swelling, expressed as a probability function. Arrows indicate the time of application of  $250 \text{ J/m}^2$  in ten separate experiments. The final percent emergence is plotted on a probability scale versus the time of UV application.

Symbols: (  ) percent emergence at the time of UV application  
(  ) percent emergence of myxamoebae from irradiated spores 6 hours after activation





point. Since a 45 sec UV exposure also resulted in less than 1% emerged myxamoebae by 24 hours, it would appear that even this low fluence may be used to determine the competence point. However, when UV exposure times below 45 seconds are used some emergence is observed (Fig. 2). The various final percent emergence levels which were observed at these low UV fluences should not be confused with the final percent emergence levels observed when germinating spores are irradiated after the competence point (Fig. 9c). The latter phenomenon is an effect of population synchrony rather than a possible effect of UV repair since there is no indication of spore recovery at the fluences used to determine the competence point. We do not exclude the possibility of some small amount of repair, not detectable by the methods employed here, which might be occurring at the very low UV fluences. It is at such low fluences that some delayed response in emergence may be observed (Fig. 5).

#### 4. Respiration of irradiated spores

Cotter and Raper (1968b) and Kobilinsky and Beattie (1977) reported that spore germination of Dictyostelium discoideum is strictly aerobic. Germination in this organism is inhibited at any stage by oxygen deprivation or by the addition of respiratory poisons such as azide (Cotter and Raper, 1968b). Thus it would be of considerable importance to determine if the application of UV light had any effect on spore respiration. Indeed, the first set of experiments conducted on UV irradiated spores were the respiration experiments. It would be of little use to examine

macromolecular requirements for the spore germination process, employing UV light as a probe, if UV irradiation were found to inhibit the respiration of Dictyostellium discoideum spores.

Irradiated dormant spores which are subsequently activated exhibit normal oxygen uptake during swelling (Fig. 10b). The respective germination kinetics are shown in Fig. 10a. The difference between control and UV irradiated spores begins to appear at about the time that emergence initiates in the control population. The cumulative  $O_2$  uptake of UV irradiated spores, (up to 5 hours) is thus about 20% less than the control value. Dormant spores exhibit an  $O_2$  uptake which is only slightly above that observed for the oxygen probe.

The data of Fig. 11 show the similar endogenous oxygen uptake of UV irradiated spores (150 sec UV exposure) compared with that of cycloheximide treated spores (200 ug/ml). The endogenous oxygen uptake of dormant spores and the probe values from Fig. 10b are also included in Fig. 11 for comparative purposes. In Fig. 11 it is again observed that spores which are inhibited from emerging exhibit a lower cumulative value for  $O_2$  uptake than control spores. A 20% reduction in  $O_2$  consumption when spores are inhibited from emerging by the addition of cycloheximide has previously been shown (Cotter et al., 1979). A combination of both UV and cycloheximide treatment resulted in values for  $O_2$  consumption which were comparable to the values for the separate application of the two treatments. It thus appears that the inhibition of  $O_2$  uptake by inhibitors of emergence is a stage specific one.

Figure 10a.

The germination kinetics of ~~activated~~ spores monitored during the respiration experiment of Fig. 10b. Spores which were irradiated received a fluence of  $250 \text{ J/m}^2$  immediately after heat activation.

Symbols: ( ○ ) percent swelling of control spores  
 ( ● ) percent emergence of myxamoebae from control spores  
 ( △ ) percent swelling of irradiated spores  
 ( ▲ ) percent emergence of myxamoebae from irradiated spores

Figure 10b.

Endogenous oxygen uptake of irradiated spores during germination.

Symbols: ( ○ ) respiration of control spores  
 ( △ ) respiration of spores exposed to  $250 \text{ J/m}^2$   
 (150 sec of UV exposure)  
 ( ● ) respiration of dormant spores  
 ( □ ) oxygen probe

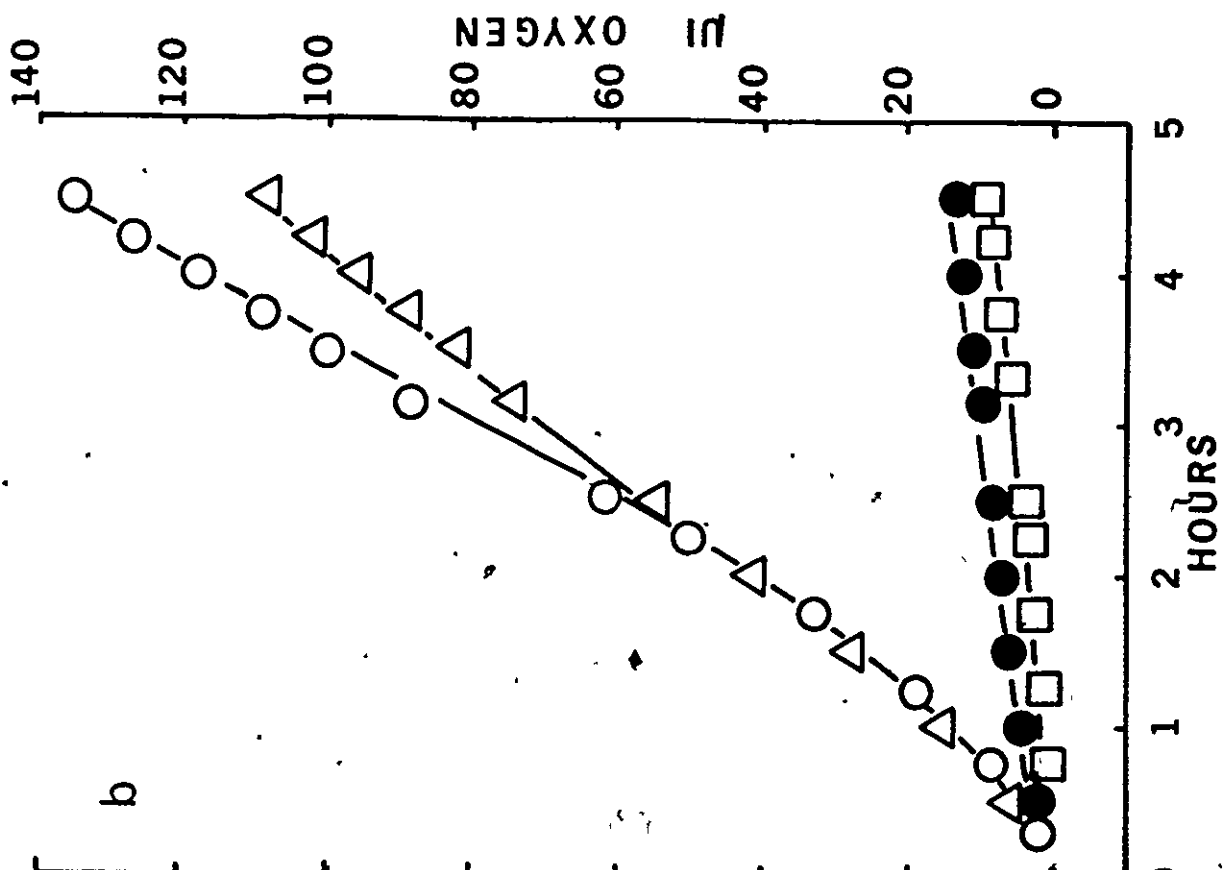
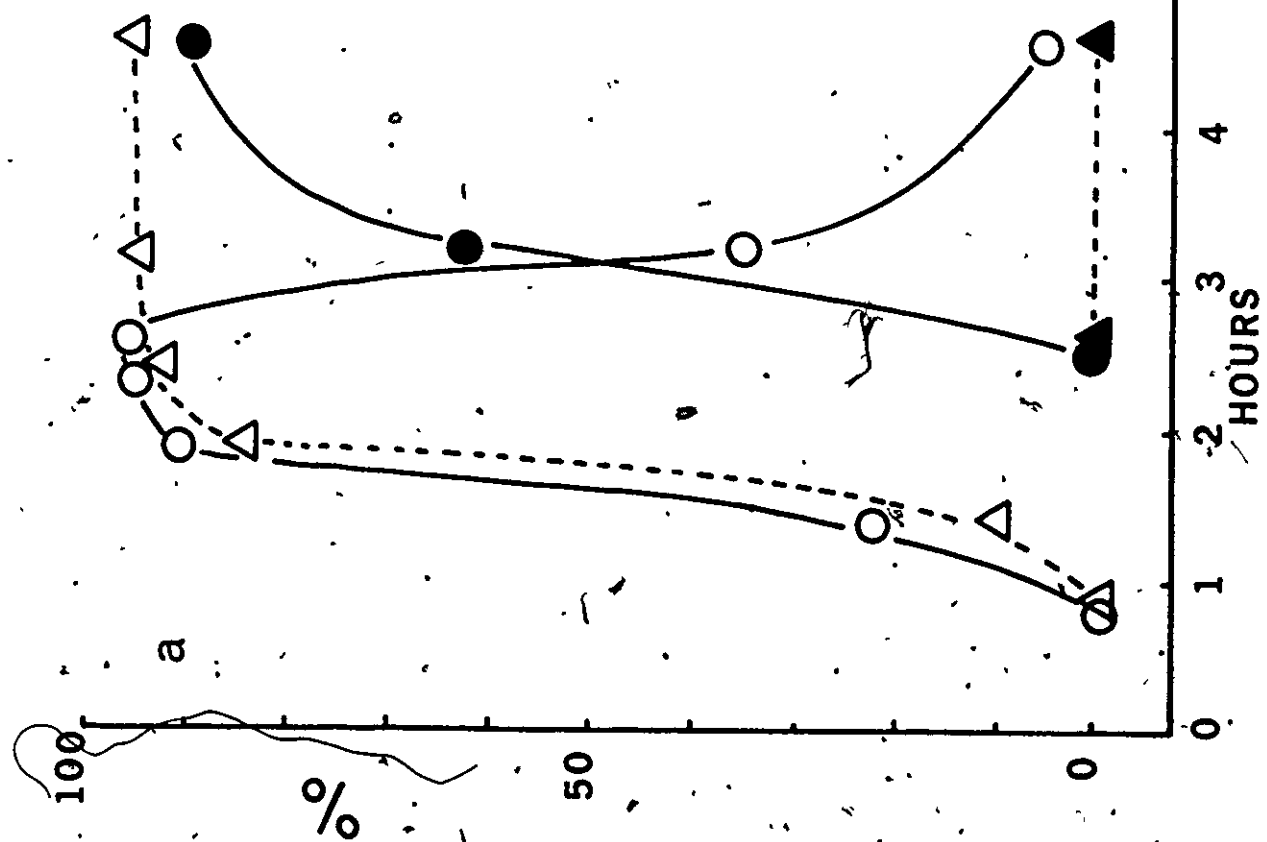
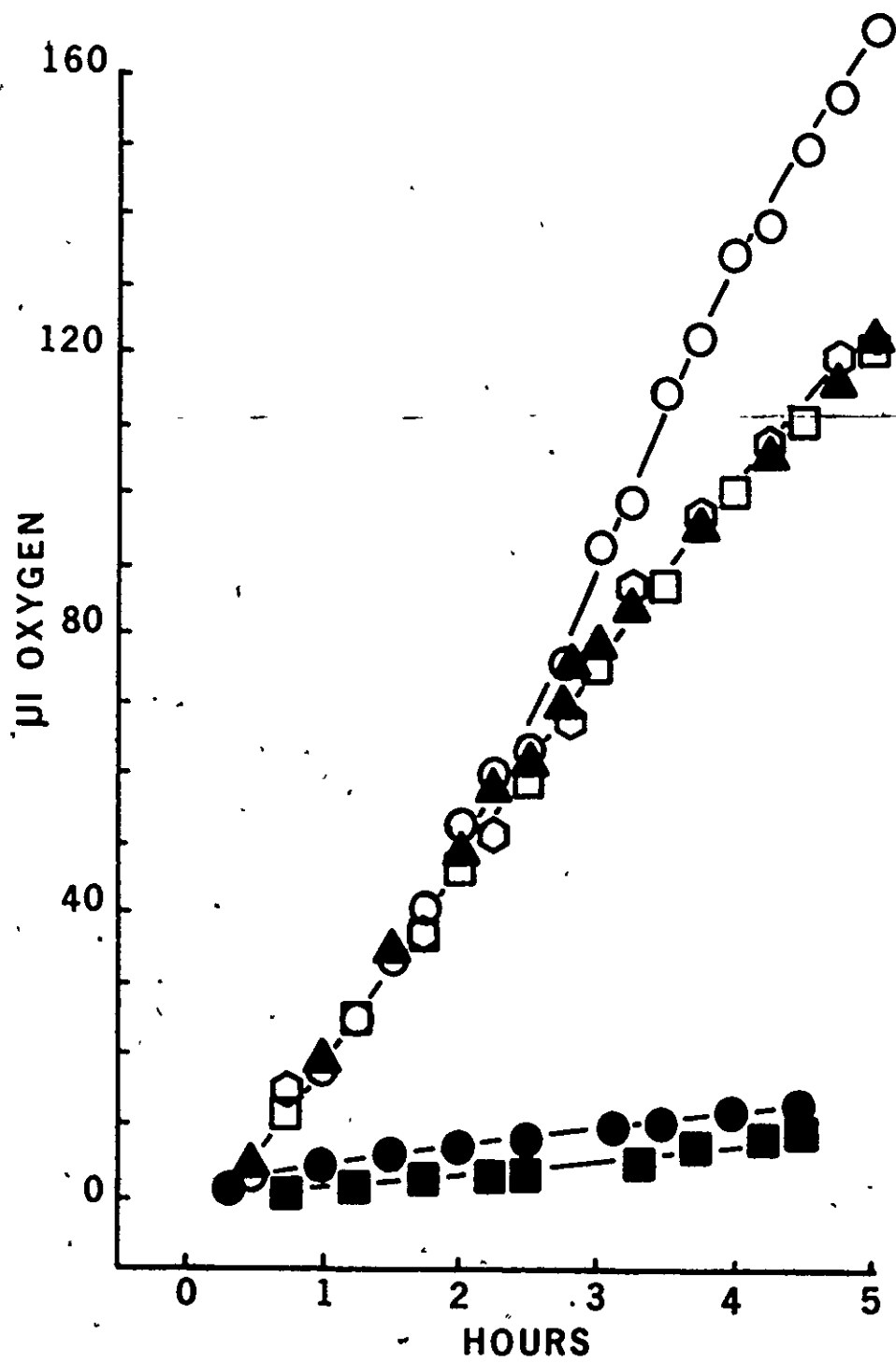


Figure 11.

Endogenous oxygen uptake of germinating spores. Emergence began in control spores at 2 1/2 hours after activation. Spores which were irradiated received a fluence of 250 J/m<sup>2</sup>.

Symbols: ( ○ ) control spores  
( ⬡ ) cycloheximide treated spores  
( ▲ ) UV irradiated spores  
( □ ) UV irradiated + cycloheximide treated spores  
( ● ) dormant spores (nonactivated)  
( ■ ) oxygen probe



Maximally swollen spores apparently consume the same relative quantity of oxygen no matter how they are inhibited from emerging; this value is less than the amount of oxygen consumed by the same quantity of emerged myxamoebae. The data plotted in Fig. 12b compare the cumulative oxygen uptake of spores exposed to 2.5 min (150 sec) of UV ( $250 \text{ J/M}^2$ ). There does not appear to be a significant difference in this particular experiment between the respiration of spores irradiated with the two UV fluences. It is noted in Fig. 12a that 5 min of UV-exposure causes a slightly greater delay in swelling of heat activated spores than 2.5 min of UV exposure. This delay in swelling is slightly variable between independent experiments and may explain the slightly lower values in  $\text{O}_2$  accumulation of some spores exposed to 5 min of UV irradiation (data not shown). A second control shown in Fig. 12b was irradiated at the time indicated by the arrow (about 2 hours after activation). The spores were removed from the vials at about 2 hours after activation, exposed to 2.5 min of UV, and then returned to the vial for continued oxygen monitoring. The second control was an attempt to determine if UV could induce an immediate cessation of respiration at the time of its application. The experiment demonstrates that the spores irradiated at 2 hours continue to consume  $\text{O}_2$  at about the same rate as prior to irradiation. However, the rate of  $\text{O}_2$  consumed in the second control does not increase as it did for the first unirradiated control. Since the spores in the second control were inhibited from releasing amoebae, it further supports the idea that the higher

Figure 12a.

Germination kinetics of activated spores monitored during the respiration experiment of Fig. 12b. Spores were irradiated at a fluence rate of  $1.7 \text{ J/m}^2/\text{sec}$  immediately after activation.

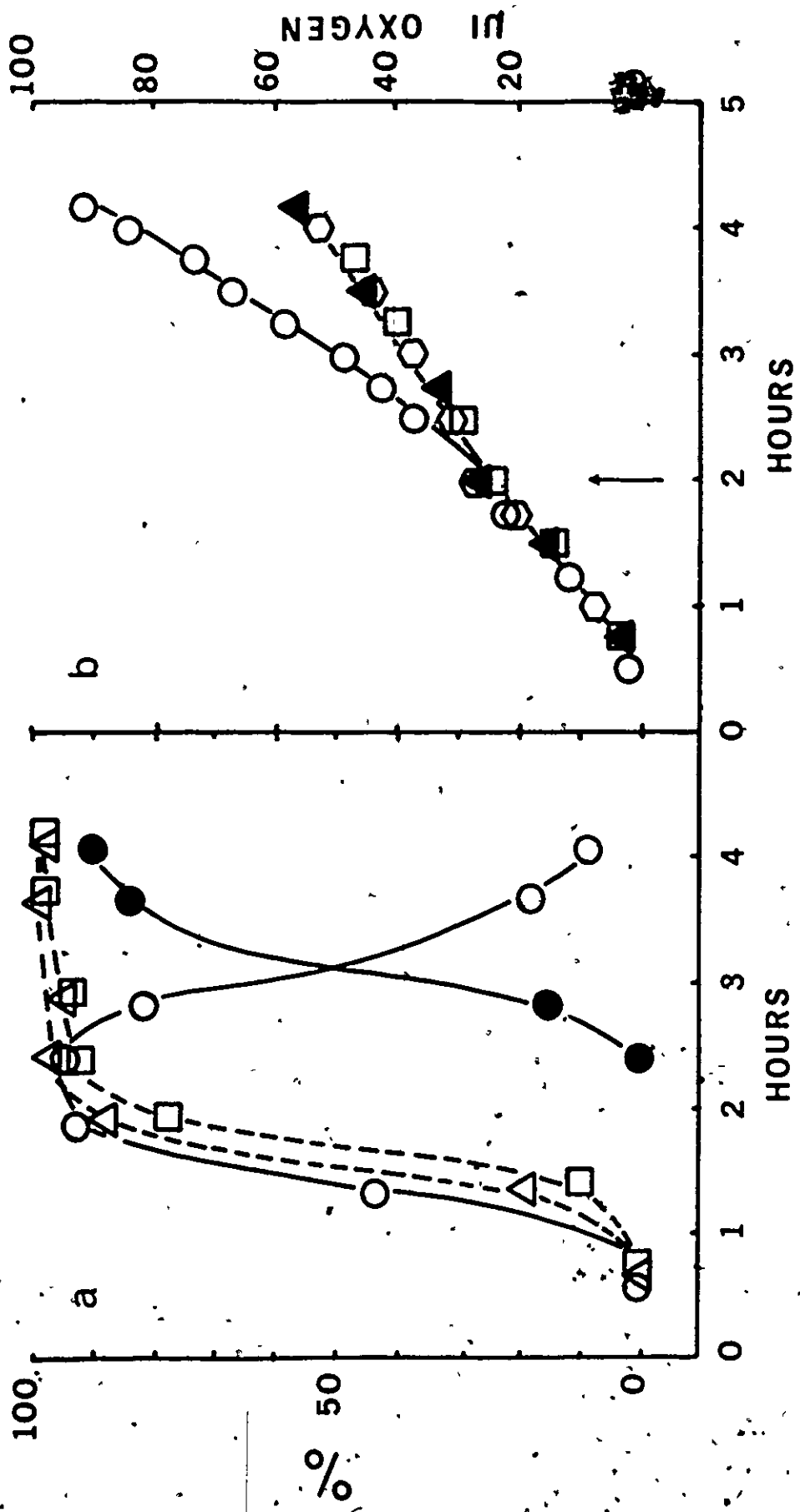
Symbols: ( ○ ) percent swelling of control spores  
 ( ● ) percent emergence of myxamoebae from control spores  
 ( △ ) percent swelling of spores exposed to 150 sec of UV  
 ( □ ) percent swelling of spores exposed to 300 sec of UV

Figure 12b.

Endogenous oxygen uptake of germinating spores of Fig. 12a. The spores were irradiated as described above.

Symbols: ( ○ ) respiration of control spores  
 ( ▲ ) respiration of spores exposed to 150 sec of UV  
 ( □ ) respiration of spores exposed to 300 sec of UV  
 ( ⬡ ) respiration of control spores exposed to 150 sec of UV at 2 hours after heat activation (as indicated by the arrow)





rate of  $O_2$  accumulation in the unirradiated control is actually a stage specific effect which is due to the emergence of myxamoebae.

5. Chemical activation and deactivation of irradiated spores

Since heat shocked UV irradiated spores responded to activation like control unirradiated spores (Fig. 7) it was expected that the former should also respond to other methods of activation. Indeed, UV irradiated spores as well as control spores were observed to swell after treatment with DMSO, urea, ethylene glycol and methanol as described in the materials and methods section. Even though the kinetics for the chemical activation methods were similar to those diagrammed in Fig. 7 (data not shown) it is apparent that the efficiency of the chemical activation methods are critically dependent on the effectiveness of the washings to free the spores from the activating chemicals (Cotter, 1981). Thus there was variation between independent experiments for the different methods of activation (data not shown). Despite the variations UV irradiated spores are activated as well as the non-irradiated controls given a particular method of activation. No emergence was observed after chemical activation of irradiated spores.

UV irradiated spores were also found capable of deactivation. Heat activated and UV irradiated spores incubated in the presence of 0.25 M sucrose for 24 hours remained unswollen even after the spores were washed free of sucrose the following day. Both control and UV irradiated spores did not begin to

swell until another heat shock was applied to reactivate the spores. The germination kinetics observed following the second heat shock were still essentially sigmoidal (Fig. 13 , Panel A) with the control spores exhibiting a somewhat longer lag for swelling than observed for the first heat shock. The longer lags for swelling after a subsequent heat activation treatment have been previously observed (Cotter and Raper, 1968b). The lag for swelling of UV irradiated spores was longer than that of the controls, but most of the irradiated spores were able to reach maximum swelling (Fig. 13 , Panel B). Again, while control spores released amoebae the UV irradiated spores did not proceed past the maximum swelling stage of germination.

In all the studies described thus far only spores of Dictyostelium discoideum NC4 wildtype were used. These spores have been shown to be constitutively dormant and do not become capable of germinating in the absence of an exogenous activation treatment until at least 1-2 weeks of maturation in the intact sorocarp (Dahlberg and Cotter, 1979). However, spontaneous mutants have been isolated which autoactivate without prior activation treatments and also without prior aging. The only requirement is that freshly harvested Sgl spores be washed free from autoinhibitor substances (Cotter and Dahlberg, 1977). In Fig. 14, the effect of UV irradiation on one such mutant (Sgl) is shown. Though the swelling kinetics of the irradiated mutant appear to be slightly altered the Sgl spores were able to autoactivate despite UV irradiation. Previous work on UV irradiation of Sgl spores has shown that in some cases the

Figure 13.

Germination kinetics of reactivated spores. Spores which were irradiated received 150 sec of UV exposure at a fluence rate of  $1.7 \text{ J/m}^2/\text{sec}$ . In Panel A, the kinetics are shown for control and irradiated spores after normal heat activation. In Panel B, the kinetics are shown for the control and irradiated spores which were deactivated and subsequently reactivated.

Symbols: ( ○ ) percent swelling of control spores  
( △ ) percent swelling of UV irradiated spores  
( ● ) percent emergence of myxamoebae from control spores

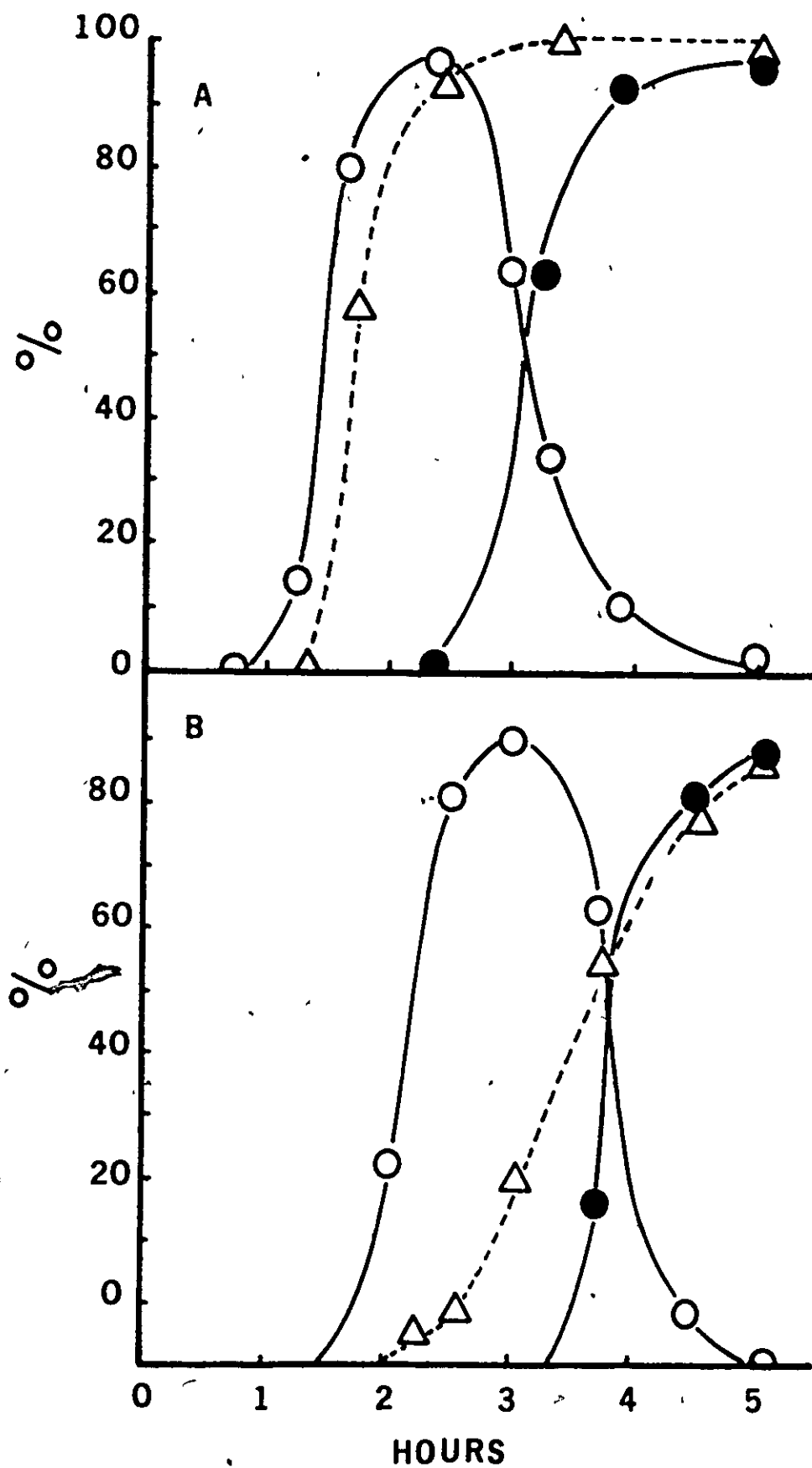
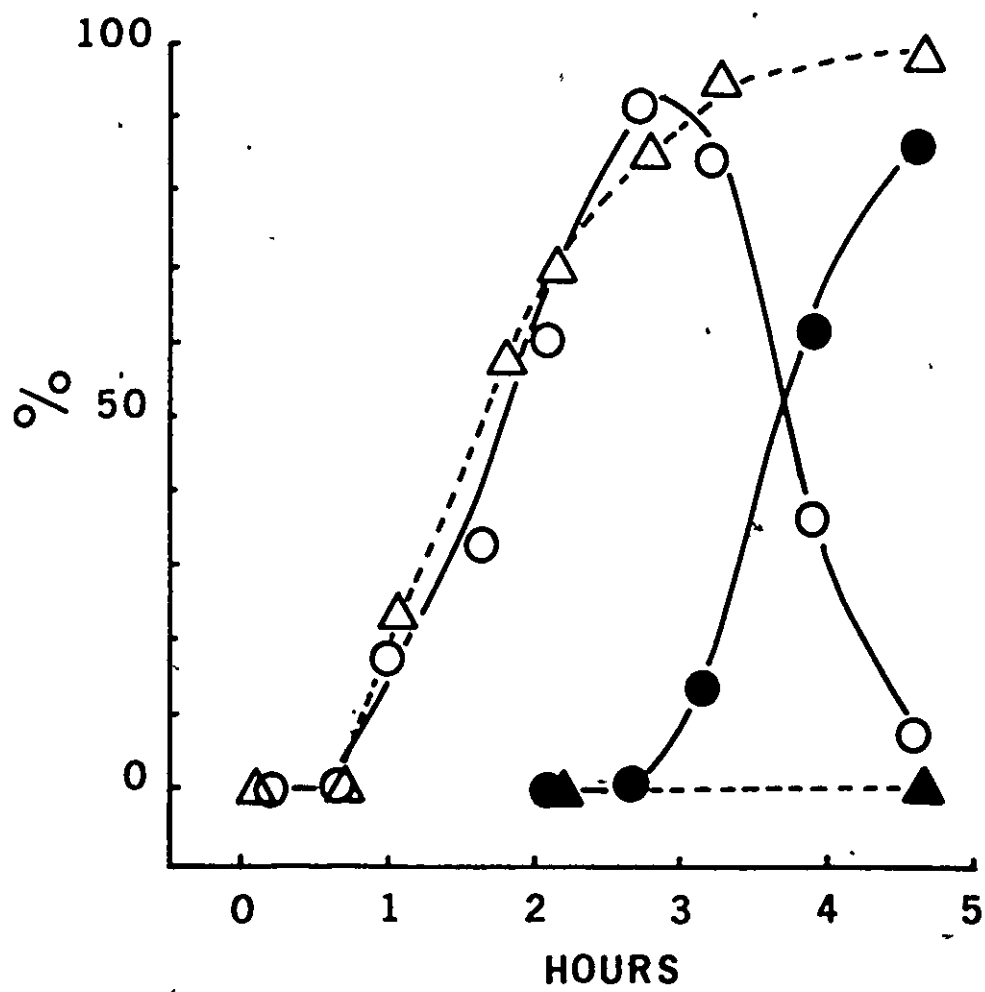


Figure 14.

Germination kinetics of autoactivated Sgl spores exposed to UV light. These spores do not require exogenous activation treatments and are simply washed free of auto inhibitor substances. Spores were irradiated for 150 sec of UV at a fluence rate of  $1.7 \text{ J/m}^2/\text{sec}$  3 minutes after the washing procedure had been completed.

Symbols: ( ○ ) percent swelling of control spores  
( ● ) percent emergence of myxamoebae from control spores  
( △ ) percent swelling of irradiated spores  
( ▲ ) percent emergence of myxamoebae from irradiated spores



spores exhibited slower swelling kinetics, (Cotter and Dahlberg, 1977). The reason for the slower swelling observed in some autoactivation experiments, and in the activation-deactivation experiments described above, is not known at the present time. Nevertheless, UV irradiation at the fluences used here does not prevent the autoactivation phenomenon though it may alter the kinetics somewhat. The emergence of myxamoebae from autoactivated spores is prevented by UV as expected.



Part II The Effects of Ultraviolet Light on Macromolecular  
Synthesis During Spore Germination of Dictyostelium  
discoideum

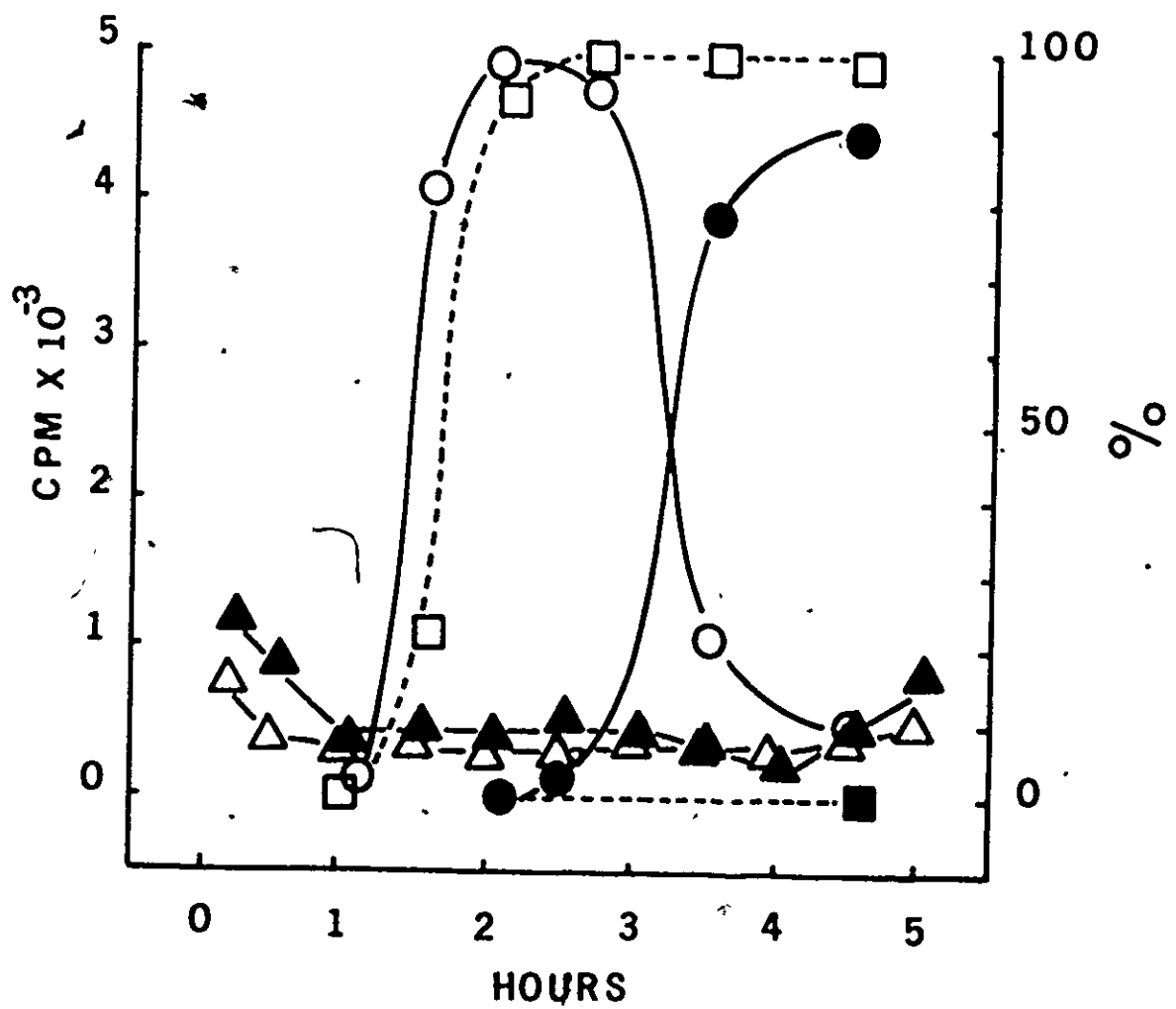
1. DNA synthesis during spore germination

In UV irradiated vegetative cells of Dictyostelium  
discoideum, the synthesis of DNA is inhibited most, followed  
by RNA synthesis and protein synthesis (Freim and Deering,  
1970). DNA synthesis in germinating spores of Dictyostelium  
discoideum was monitored by the incorporation of methyl ( $^3\text{H}$ )-  
thymidine (10 Ci/mmole, ICN) at 5  $\mu\text{Ci/ml}$ . As can be seen in  
Fig. 15, there was no apparent accumulation of radiolabelled  
thymidine into TCA insoluble precipitates of irradiated or  
non-irradiated germinating spores of Dictyostelium discoideum.  
These heat-activated spores exhibited normal swelling and  
emergence in the 5 hour time period during which thymidine  
incorporation was evaluated. This result is contrary to that  
of Yagura and Iwabuchi (1976) who reported that thymidine in-  
corporation begins when the germinating spores enter the  
emergence phase (between 2 and 3 hours after activation).  
The reason for this discrepancy is not known. There have  
been no other similar reports in the literature of thymidine  
incorporation occurring during the germination of Dictyo-  
stelium discoideum spores. In general, however, it has been  
shown in other germinating fungal systems such as Botrodiplo-  
dia theobromae and Rhizopus stolonifer B for example, that  
DNA synthesis begins after the initiation of RNA and protein  
synthesis (Dunkle and Van Etten, 1972). These workers

Figure 15.

Thymidine incorporation during spore germination of Dictyostelium discoideum. Spores were irradiated at a fluence of  $250 \text{ J/m}^2$  immediately after heat activation. The precursor methyl ( $^3\text{H}$ )-thymidine (10 Ci/mmolé, ICN) was used at 5  $\mu\text{Ci/ml}$ , and added immediately after the UV treatment.

- Symbols:
- ( ○ ) percent swelling of control spores
  - ( ● ) percent emergence of myxamoebae from control spores
  - ( □ ) percent swelling of irradiated spores
  - ( ■ ) percent emergence of myxamoebae from irradiated spores
  - ( △ ) incorporation of control spores
  - ( ▲ ) incorporation of irradiated spores



concluded that DNA replication was not required for early transcription. The tentative conclusion from results with DNA synthesis inhibitors was that DNA synthesis was not essential for germination (Van Etten et al., 1976).

One possibility which was considered as an explanation for lack of detection of thymidine incorporation during spore germination was that methyl ( $^3\text{H}$ )-thymidine may be a poor precursor for DNA synthesis in Dictyostelium discoideum spores. This has been shown to be the case in some other systems; for example, an organism such as Neurospora crassa which lacks thymidine kinase incorporates about 10 times more radioactivity into its RNA than DNA when  $2(^{14}\text{C})$ -thymidine is used as a radioactive precursor (Grivell and Jackson, 1968). Grivell and Jackson (1968) reported that thymidine kinase activity is present in some organisms but not in others. Another example can be seen in the protoplasts of the prokaryote (cyanobacterium) Anacystis nidulans. Unlike the intact cells of A. nidulans, which are apparently impermeable to exogenously supplied nucleic acid precursors, the protoplasts can incorporate radioactive thymidine (Pigot and Carr, 1971). However, these protoplasts apparently lack thymidine kinase activity and like N. crassa most of the exogenously supplied precursor (90% of  $2(^{14}\text{C})$ -thymidine is located in the RNA rather than the DNA (Restaino and Frampton, 1975). It has recently been found that thymidine kinase activity is present in the soluble and the mitochondrial fractions of axenically grown Dictyostelium discoideum cells (Michrina and Deering, 1980). It has also

been reported that exogenous thymidine can be incorporated into both nuclear and mitochondrial DNA's of Dictyostelium discoideum vegetative cells (Firtel and Bonner, 1972). Moreover, Deering and co-workers have successfully used (<sup>3</sup>H)-thymidine labelled E. coli to radioactively label the DNA of Dictyostelium vegetative cells for pyrimidine dimer analysis and alkaline sucrose gradient density centrifugation (Guialis and Deering, 1976a, b; Welker and Deering, 1979a, b; Ford and Deering, 1979; Kielman and Deering, 1980; Clark and Deering, 1981). Thus it would appear that thymidine is a suitable precursor for the radioactive labelling of the vegetative cells of Dictyostelium discoideum. There is however a question of the permeability of spores to exogenously supplied label. Dictyostelium spores have been found impermeable to some compounds (Giri and Ennis, 1977; Cotter, 1977). It is apparent from the uracil and leucine incorporation data which are to follow, that germination spores of Dictyostelium discoideum are permeable to label, at least during the swelling stage of germination.

Thus, in considering these possibilities, it would seem that the incorporation data of Fig. 15 are indeed reflecting a lack of DNA synthesis during the spore germination phase of the Dictyostelium discoideum life cycle. The fact that DNA synthesis may be absent in germinating spores of Dictyostelium was first postulated by Cotter (Ph.D Thesis) who compared the DNA content of spores with that of emerged myxamoebae. The DNA of both cell types was relatively the same,

and thus no significant DNA synthesis could have taken place during the transition from the quiescent to the actively growing state. In any case, it has been observed by Cotter and other researchers, that every spore releases only a single myxamoebae, and thus cell division does not take place prior to emergence. There is therefore no necessity for DNA synthesis at this time. Nevertheless, the data on this matter is still minimal and further work is necessary.

There are examples of other germinating systems in which DNA synthesis does not occur during the transition from the dormant to the vegetative state. For example, ( $^3\text{H}$ )-thymidine is not incorporated into acid precipitable macromolecules during the first 6 hours after germination is initiated in the microcysts of the slime mold, Polysphondylium pallidum (O'Day et al., 1976). O'Day and co-workers concluded that DNA replication does not begin until some time after the first 6 hours of germination and therefore DNA synthesis is not essential for germination. Another example in which DNA synthesis does not occur is during the germination of Petunia hybrida pollen; it has been observed that little or no DNA synthesis occurs before division of the generative cell in the elongating tube of the germinating pollen (Jackson and Linskens, 1979). This germinating pollen system has been described as a convenient system with which to monitor UV-induced unscheduled DNA repair synthesis, since normal semi-conservative DNA replicative synthesis is absent. The unscheduled labelling of DNA by radiolabelled thymidine was

believed to represent DNA repair synthesis necessary to fill gaps present after thymine dimer excision in UV irradiated pollen. The data presented in Fig. 15 suggest that UV irradiated spores of Dictyostelium discoideum did not exhibit an "unscheduled" incorporation of radiolabelled thymidine. This may mean that in addition to the absence of normal replicative DNA synthesis, germinating spores of Dictyostelium discoideum also do not exhibit repair synthesis after UV irradiation. It is possible that any repair synthesis that may be occurring after UV irradiation of spores is so low that it can not be detected by the means of monitoring in a continuous labelling experiment such as that of Fig. 15. Repair synthesis, at low fluences has been monitored in bacterial systems by a density labelling technique using the thymine analogue, 5-bromouracil. It was reported that a fluence of  $50 \text{ J/m}^2$ , which in the case of E. coli led to a  $4 \times 10^{-2}$  survival of colony formers, was appropriate for the density-labelling studies (Pettijohn and Hanawalt, 1964). However, these workers were using repair proficient bacterial strains in which it would be easier to detect repair synthesis at the lower fluences. Presently, it does not seem that Dictyostelium discoideum spores would be amenable to such analysis, since it is difficult to specifically label the DNA of this organism (Michrina and Deering, 1980) and also, it may not be possible to extract spore DNA without a significant amount of sheering. For example, Ford and Deering (1979) have reported that once final differentiation into

spore and stalk cells commenced, lysis of cells and alkaline sucrose gradients became difficult and hence gradient analysis was not performed at later times in development.

2. The effect of ultraviolet light on RNA synthesis during germination of Dictyostelium discoideum spores

The inhibitory effect of UV light on RNA synthesis has been reported in numerous systems (see literature review). Early UV studies have also shown that irradiation causes a retardation in the synthesis of ribonucleic acid synthesis and protein synthesis for Dictyostelium discoideum vegetative cells (Freim and Deering, 1970). The effect of UV on RNA and protein was somewhat less than its effect on DNA. The main physiological effect observed in these initial UV experiments by Deering was a UV-induced division delay of vegetative cells which he mainly attributed to the inhibition of DNA synthesis. However, the synthesis of all three macromolecular classes tested by Deering were observed to resume before the period of division delay. Thus, UV did not present the vegetative cells with a permanent inhibition of macromolecular synthesis. Apparently the vegetative cells were capable of repair of a considerable amount of the UV induced damage (as discussed in the literature review). The situation is markedly different for irradiated spores of Dictyostelium discoideum. It has been shown in Part I of this thesis (Fig. 4 and Fig. 6) and by Cotter (1973) that UV irradiation of spores completely inhibits the emergence stage of germination. Thus it was of considerable importance



to examine if RNA synthesis in spores was also as severely inhibited as was the emergence phenomenon.

RNA synthesis in non-irradiated and irradiated Dictyostelium discoideum spores was evaluated by the incorporation of (<sup>3</sup>H)-Uracil at 5 uCi/ml (25 Ci/mmmole, NEN and Amersham). The incorporation of (<sup>3</sup>H)-Uracil into TCA insoluble precipitates begins sometime during the swelling phase of germination, and increases rapidly just prior to emergence (Fig. 16a). This has been previously observed by other researchers (Giri and Ennis, 1977a, b; Yagura and Iwabuchi, 1976). However, the level of radioactivity accumulated in irradiated spores by 5 hours of germination is only about 10% that of control values. (The levels observed in Fig. 16a were from 1 independent experiment, and were somewhat higher than that observed in other typical experiments.)

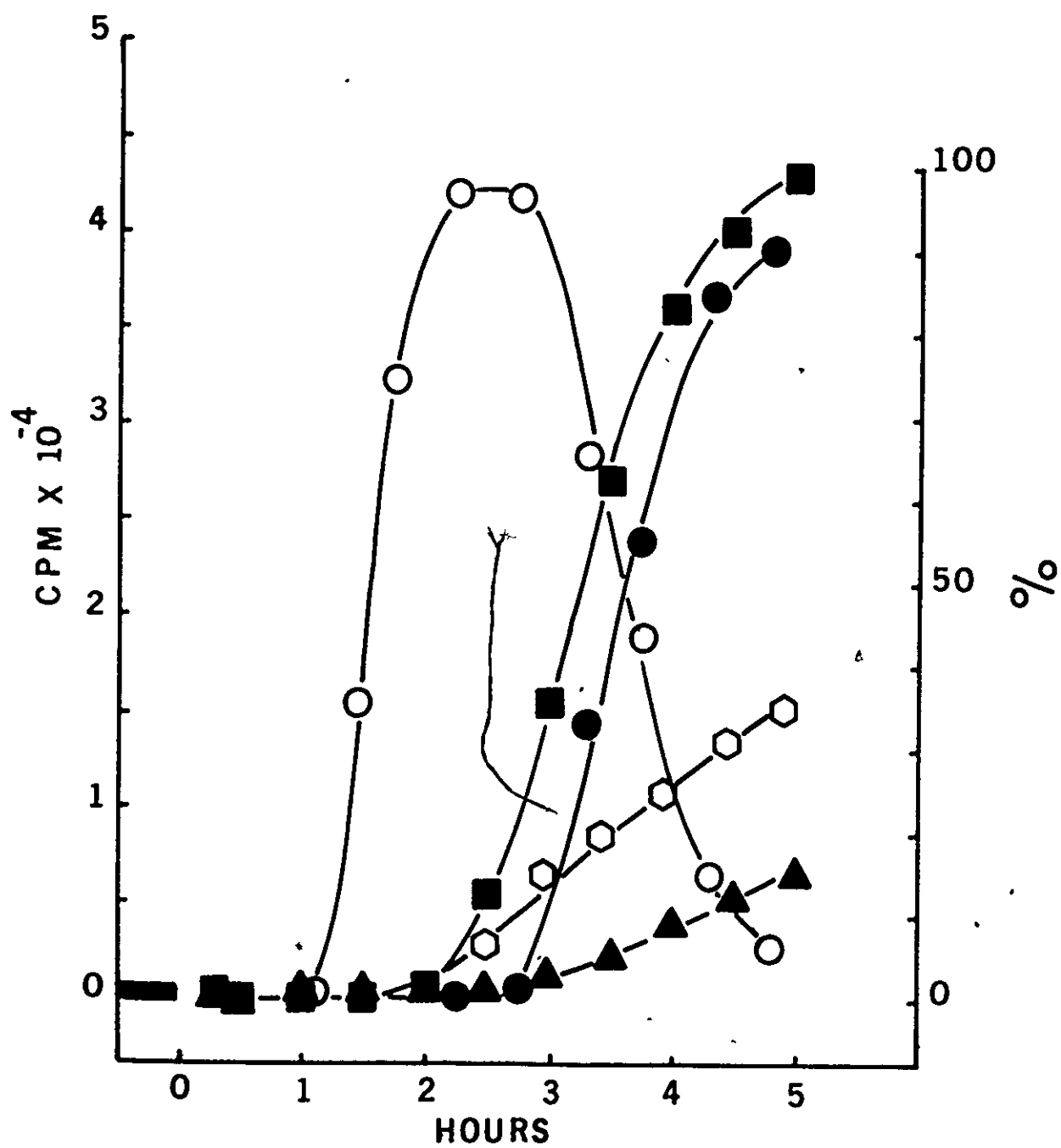
Cycloheximide, a protein synthesis inhibitor (Siegel and Sisler, 1964) is also observed to reduce the incorporation of radiolabelled uracil into germinating Dictyostelium discoideum spores (Fig. 16a). (This phenomenon has been observed previously for Dictyostelium by Giri and Ennis (1978) ). The possible mechanism for the apparent inhibition of RNA synthesis in cycloheximide treated spores will be considered later in this thesis. As discussed in the literature review, a number of chemical inhibitors (of RNA synthesis) have been employed to probe the possible requirements for RNA synthesis during spore germination. However, the use of chemical inhibitors of spore macromolecular

Figure 16a.

The effect of UV light and cycloheximide on the incorporation of radiolabelled uracil during spore germination. Spores were irradiated with a total fluence of  $250 \text{ J/m}^2$ , or suspended in cycloheximide (200 ug/ml), immediately after heat activation. The RNA precursor ( $^3\text{H}$ )-uracil (25 Ci/mmmole, Amersham) was used at 5 uCi/ml.

Symbols: (■) period of heat activation  
 (○) percent swelling of control spores  
 (●) percent emergence of myxamoebae from control spores  
 (■) control incorporation  
 (▲) incorporation of UV irradiated spores  
 (⬡) incorporation of cycloheximide treated spores

The swelling of UV irradiated spores was only slightly delayed as shown in previous experiments. The emergence of both cycloheximide and UV irradiated spores was completely inhibited.



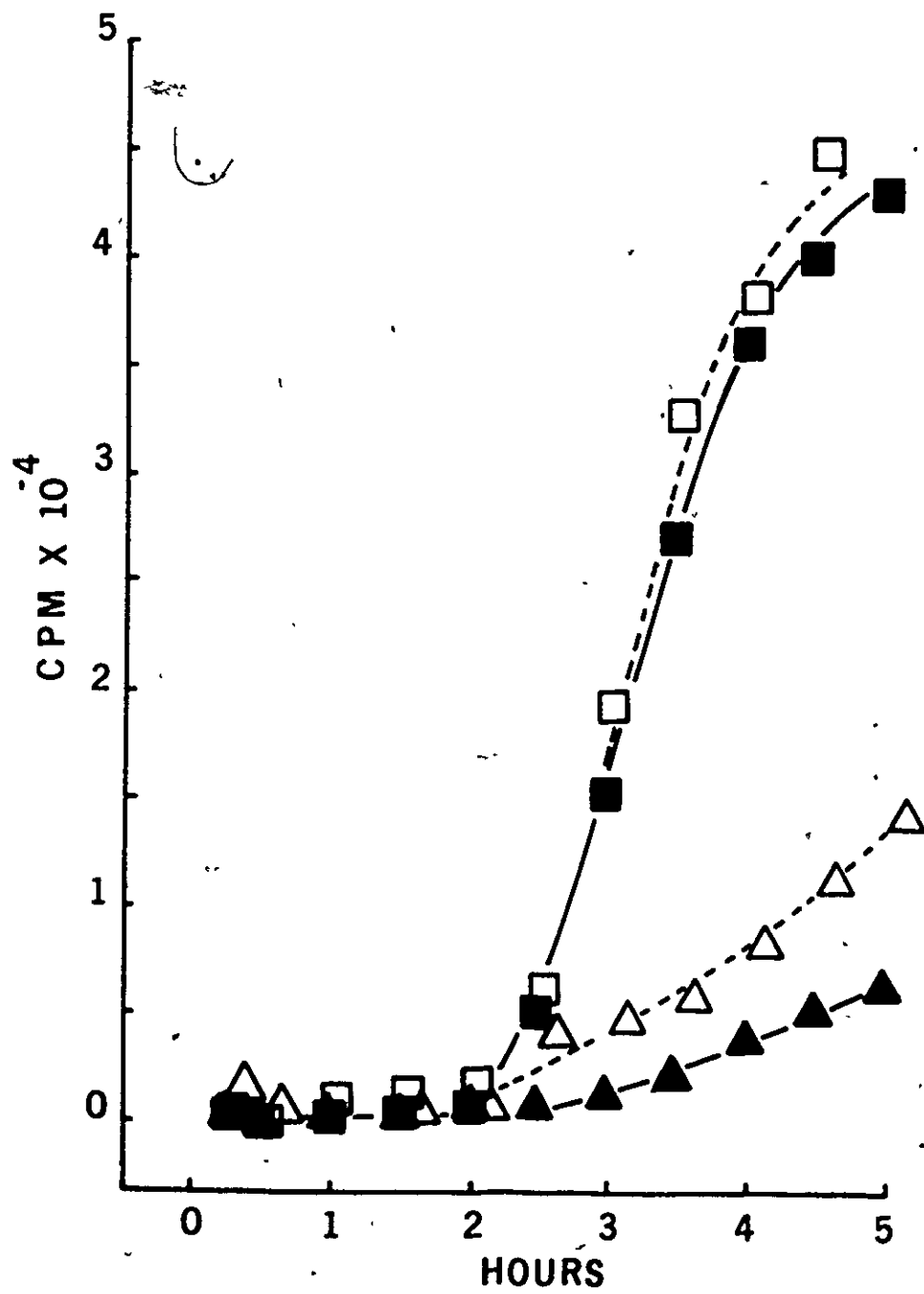
synthesis has always been plagued with permeability problems (Van Etten et al., 1974). The problem of inhibitor permeability is overcome with the use of a physical inhibitor such as UV light. The data of Fig. 16a, using UV as the inhibitor, would suggest that UV light is acting as an effective block to RNA synthesis as measured by precursor incorporation. However, it has also been cautioned that a definite conclusion regarding RNA synthesis cannot be made from incorporation data alone without a knowledge of precursor pools (Giri and Ennis, 1977; Cotter et al., 1979). The possibility that UV may be inhibiting precursor uptake must also be considered since there have been reports of the inhibition of precursor transport by UV in some systems (Ascenzi and Jagger, 1979). Much of the permeability effects however, were induced by UV in the range of 360nm to 400nm (near UV) as opposed to the far UV (254nm) used in these studies. It is in the near UV range of the electromagnetic spectrum that enzyme molecules may tend to absorb the radiation more than the nucleic acids and therefore one may be observing UV inactivation of a membrane permease for example (Doyle and Kubitschek, 1976). Nevertheless, it would still appear to be useful to compare the total precursor accumulation (uptake), relative to that which is actually incorporated into the macromolecules of the spores. The data of Fig. 16b which was obtained in the same experiment as Fig. 16a was plotted separately for the purpose of clarity. The dashed line representing precursor uptake can be seen to correspond very closely to the solid line representing precursor incorporation for nonirradiated spores (almost 1:1). This might suggest that exogenous label which is taken up is incorporated

Figure 16b.

Comparison of uracil uptake and incorporation for control and UV irradiated spores during germination. Spores were irradiated with a total fluence of  $250 \text{ J/m}^2$  immediately after heat activation. The RNA precursor ( $^3\text{H}$ )-uracil (25 Ci/mmol, Amersham) was used at 5 uCi/ml.

Symbols: (  $\square$  ) uptake of control spores  
(  $\blacksquare$  ) incorporation of control spores  
(  $\triangle$  ) uptake of irradiated spores  
(  $\blacktriangle$  ) incorporation of irradiated spores

The germination kinetics for this experiment were as described for Fig. 16a. Total uptake (dashed lines) was evaluated after phosphate buffer washes of spores, while incorporation (solid lines) was evaluated by the TCA precipitable counts from aliquots of spore suspension which were simultaneously sampled during the germination period.







immediately. In the case of UV irradiated spores the total number of counts incorporated is lower than the total number of counts accumulated due to uptake. The uptake levels for the spores inhibited at the swelling stage are also considerably lower than the uptake levels observed for emerging myxamoebae.

The effect observed for uptake in Fig. 16b might be misinterpreted as a UV effect on membrane transport; however, the data of Fig. 16c, which is also from the same experiment but expanded for the sake of clarity, would suggest otherwise. It is evident from Fig. 16c that spores which are inhibited at the swollen stage by cycloheximide also exhibit uptake values for the emerging myxamoebae. The cycloheximide incorporation values correspond very closely to the cycloheximide uptake values (1:1). This would again suggest that any exogenous label which is taken up is immediately incorporated. Note that the uptake values for both cycloheximide and UV inhibited spores are comparable (Fig. 16c). Thus the precursor accumulation that is observed may be a stage specific effect; i.e., emerging myxamoebae may have a greater capacity for precursor accumulation than spores inhibited at maximum swelling. Perhaps in amoebae more exogenous label could enter and expand the precursor pools. Spores inhibited at the swollen stage may not be able to accumulate label to such a great extent and thus less is incorporated as well. Irradiated spores however incorporate noticeably less label than they are apparently capable of accumulating, and this would

Figure 16c.

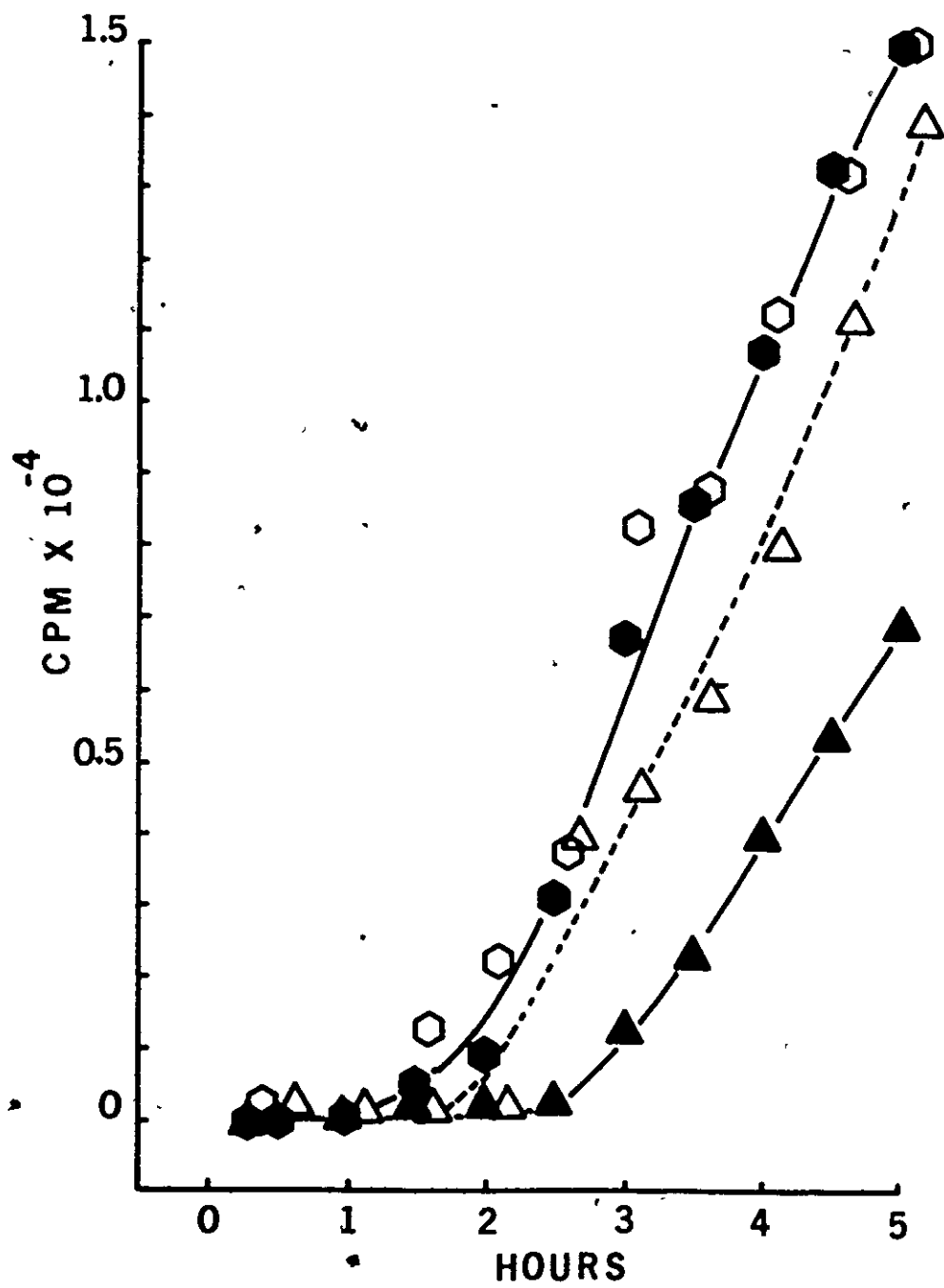
Comparison of uracil uptake and incorporation for cycloheximide treated and UV irradiated spores during germination.

\* Spores were irradiated with a total fluence of  $250 \text{ J/m}^2$  or suspended in cycloheximide (200 ug/ml) immediately after heat activation.

Symbols: (  ) uptake of cycloheximide treated spores  
(  ) incorporation of cycloheximide treated spores  
(  ) uptake of UV irradiated spores  
(  ) incorporation of UV irradiated spores

The germination kinetics for this experiment were as described for Fig. 16a. Total uptake and incorporation were performed as described for Fig. 16a and Fig. 16b. The ordinate axis was expanded to facilitate comparison of the total counts which were relatively lower in spores inhibited at the maximally swollen stage.





then appear to be due to the effect of UV inhibition of macromolecular synthesis. Though the stage specific effect may not completely explain the uptake data it may be an influencing factor. It is also not known if there is a considerable difference in the permeability of swollen spores as compared to emerged myxamoebae which might also influence the amount of label which is able to accumulate in the cell.

The mechanisms of solute transport have not been studied in great detail for Dictyostelium discoideum vegetative cells and there have been no reports of transport capabilities of germinating spores. K. C. Lee (1972) examined the permeability of Dictyostelium discoideum vegetative cells towards amino acids and inulin and concluded that the uptake of glutamate and lysine was passive while inulin was taken up actively, probably by pinocytosis. Lee also observed a "leakage" of metabolites and suggested that amoebae had no active mechanism for retaining them. Thus, since amoebae (and possibly spores) have no mechanism for active uptake and retention of amino acids and nucleotides, according to K.C. Lee, then the simplest explanation is that the cells require catabolism of protein and RNA to maintain metabolite concentrations in the pool at levels compatible with survival. Lee further suggests that since Dictyostelium discoideum cells can simply engulf bacteria, they would not be handicapped by the absence of active transport for amino acids. It has also been suggested that the permeability of spores

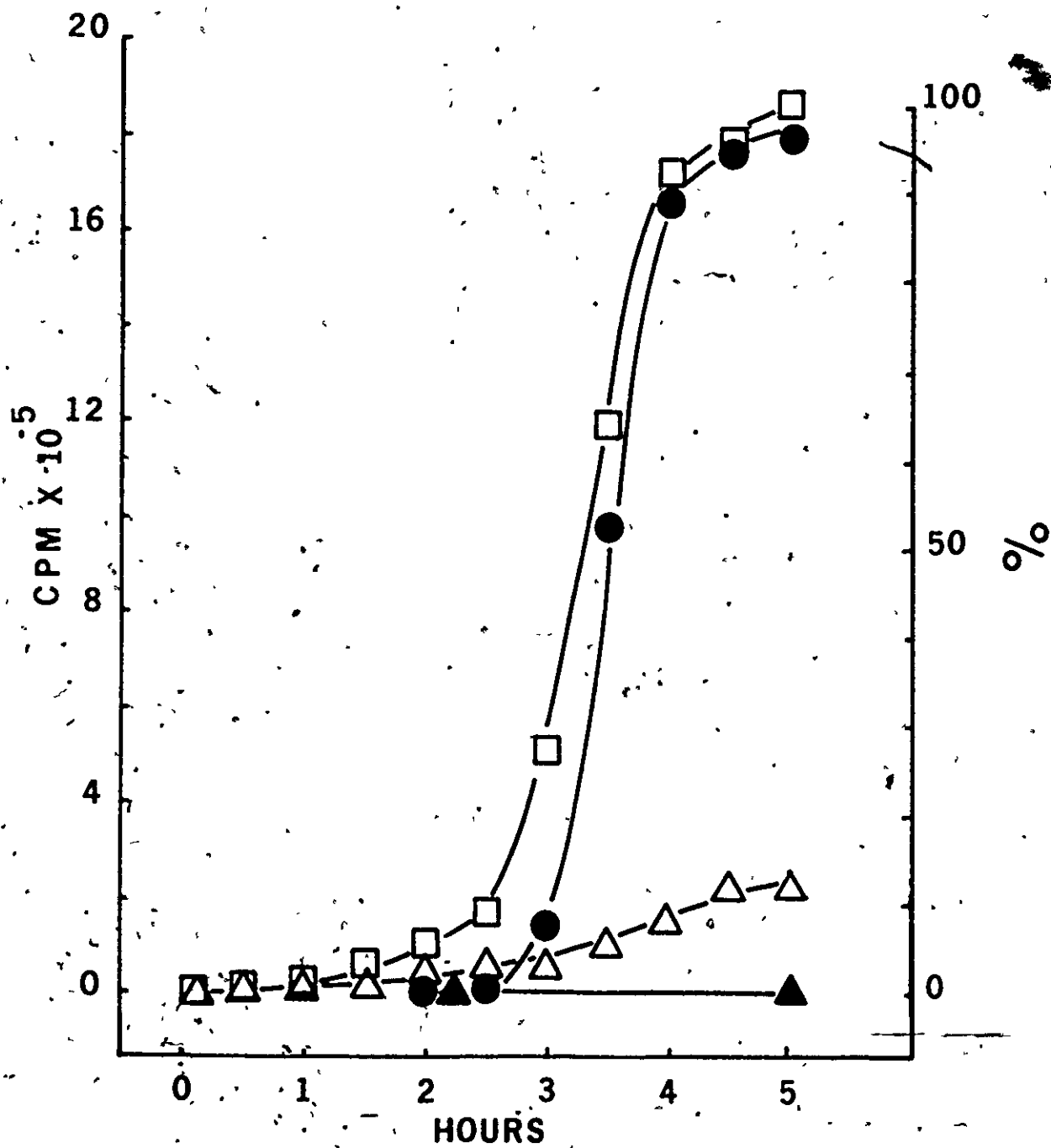
is increased following heat activation (Hohl et al., 1978) and that there is an efflux of radioactive label observed within 30 minutes after activation. Hohl et al., in 1978 had also postulated that leakage may occur in swollen spores due to an increase in osmotic pressure occurring within the spore. Thus, possible mechanisms for a loss in accumulated radioactive label in both vegetative cells and spores have been proposed and may also be considered in the interpretation of the uptake data observed in Fig. 16b and Fig. 16c.

It has been shown in the data of Fig. 16a that UV light inhibits the incorporation of radiolabelled RNA precursor. The fluence used in this experiment was about  $250 \text{ J/m}^2$  (2.5 min exposure). It has also been shown in Part I (Fig. 2) of this thesis that fluences as low as  $77 \text{ J/m}^2$  can completely prevent emergence (45 sec exposure). If it is the inhibition of RNA synthesis by UV that results in the inhibition of emergence, then a fluence of  $77 \text{ J/m}^2$  should also inhibit precursor incorporation. In Fig. 17 the data represents spores that were exposed to 45 sec of UV immediately after activation. It can be seen that 45 sec of UV is equally inhibitory to uracil incorporation as is 2.5 min ( $250 \text{ J/m}^2$ ). Thus, there is a range of UV fluences which will be equally effective in inhibiting RNA synthesis as well as myxamoebae emergence. In both Fig. 16a and Fig. 17 the data suggest that some residual precursor incorporation is occurring. It has been postulated that this may represent label which is incorporated into prematurely terminated transcripts (Giorno,

Figure 17.

The effect of a minimal UV fluence on the incorporation of radiolabelled uracil during spore germination. Spores were irradiated with a total fluence of  $77 \text{ J/m}^2$  (45 sec of UV exposure) immediately after heat activation. The RNA precursor ( $^3\text{H}$ )-uracil (25 Ci/mmol, NEN) was used at  $10 \text{ uCi/ml}$ .

Symbols: ( ● ) percent emergence of myxamoebae from control spores  
 ( ▲ ) percent emergence of myxamoebae from irradiated spores  
 ( □ ) uracil incorporation of control spores  
 ( △ ) uracil incorporation of irradiated spores



1979a, b). Such transcripts have been described as unstable (Giorno, 1979a, b) and would not be detected on routine polyacrylamide gel electrophoresis. Such transcripts would also not be capable of being transcribed into functional proteins (Giorno, 1979a, b). The consequences of such an effect will be considered in Part III of this thesis.

It was noted that the protein synthesis inhibitor, cycloheximide, had some inhibitory effect on uracil incorporation (Fig. 16a). Giri and Ennis (1978) observed a similar lower rate of ( $^3\text{H}$ )-uracil incorporation into RNA of spores which were incubated in the presence of cycloheximide. Iwabuchi and co-workers (1971) had also observed a reduced rate of ( $^{14}\text{C}$ )-uracil incorporation in Dictyostelium discoideum cells in the presence of cycloheximide. Their results suggested that 26s rRNA formation was inhibited and there was no accumulation of 28s rRNA. They concluded that the processing of the 28s molecule to the 26s rRNA was greatly suppressed during the inhibition of protein synthesis. Even earlier than this Sussman (1966) showed significant changes in the state of RNA synthesized in the presence of cycloheximide. The reports on the effect of cycloheximide on RNA synthesis in Dictyostelium discoideum have been interpreted differently by the independent researchers. However, it was as early as 1963 that cycloheximide was demonstrated by Siegel and Sisler, "to affect protein synthesis by inhibiting the transfer of amino acids from soluble RNA to the ribosomes and their subsequent polymerization into protein." It has

been postulated by a number of researchers that in various systems cycloheximide may still be acting as a protein synthesis inhibitor as shown by Siegel and Sisler, but that the inhibition of protein synthesis may be having an indirect effect on the synthesis of RNA. For example, Kloet (1966) concluded that cycloheximide interfered severely with the mechanism by which the synthesis of RNA is regulated in yeast. Kloet (1966) had postulated that the gradual deceleration of RNA synthesis in the yeast Saccharomyces carlbergensis, in the presence of cycloheximide, might be due to the fact that there may be a requirement for continuous resynthesis of a protein or a labile polymerase for normal RNA function. Timberlake and Griffen (1974) also noted that cycloheximide inhibited both protein and RNA synthesis in Achyla. They concluded that the inhibition of protein synthesis alone was not an adequate explanation for this effect. Previously, Timberlake and co-workers (1972) had observed that RNA polymerase I was inhibited in vitro by cycloheximide, and they had postulated that there was a rapidly turned over subunit of RNA polymerase I that required resynthesis. Since this model did not sufficiently explain all their results Timberlake proposed a somewhat more complex model in which the synthesis of an unknown compound (A\*) was needed for continued rRNA synthesis. The continued synthesis of the compound (A\*) was dependent on protein synthesis, and if this compound was not present in high enough concentration

then the RNA polymerase would also not function properly. It has also been observed that cycloheximide appears to inhibit the increase in the incorporation of ( $^3\text{H}$ )-uridine into total acid-precipitable material when added at 0 hour to germinating microcysts of Polyphondylium pallidum (O'Day et al., 1976). O'Day and co-workers also concluded that it was possible that the inhibition they observed was due to the prevention of synthesis of certain proteins required for the initiation of RNA synthesis (Gwynne and O'Day, 1978).

Although there is no conclusive data with which to make definite conclusions regarding the effect of protein synthesis inhibition on RNA synthesis in Dictyostelium, it may be possible to explain the data with mechanisms proposed by Timerlake for Achyla RNA synthesis or by Kloet for yeast RNA synthesis (Fig. 16a).

Additional evidence for the possible effect of cycloheximide on RNA synthesis in Dictyostelium will be presented in the following paragraphs of this thesis.

### 3. Emergence of spores released from cycloheximide inhibition

Heat activated spores which are incubated in the presence of cycloheximide (200 ug/ml) swell normally but do not release myxamoebae (Cotter and Raper, 1970). This inhibition of emergence by cycloheximide is a reversible phenomenon. Spores which are washed free of the drug via centrifugation and then resuspended in phosphate buffer will emerge (Cotter and Raper, 1970). The emergence of spores after release from



cycloheximide inhibition is relatively more synchronous than that observed for emergence of spores after heat activation. Though there is still about 1/2 to 1 hour lag after cycloheximide release, the emergence process is completed much sooner.

Spores which are irradiated prior to the removal of cycloheximide do not release myxamoebae (Fig. 18). The arrow in Fig. 18 indicates the time of application of UV light to spores incubated in the presence of cycloheximide. Application of UV to cycloheximide treated spores at about 3 1/2 hours after activation (control spores have almost completed emergence) resulted in 0% emergence even 24 hours after cycloheximide removal. (Cycloheximide was removed immediately after irradiation.) Similarly, the application of UV light to cycloheximide inhibited spores at 5 hours and later after activation, resulted in 0% emergence following cycloheximide removal (data not shown).

Spores which are irradiated immediately after the removal of cycloheximide still do not exhibit any emergence (Fig. 18). However, the application of UV at a critical time after cycloheximide removal will allow emergence to occur in a certain fraction of the population. Fig. 19a shows 8 separate applications of UV. The first three UV applications (at 3, 12, and 21 minutes after cycloheximide removal) completely inhibited emergence for at least 24 hours. However, subsequent applications of UV (at 39, 60, 69, 96, and 121 minutes after cycloheximide removal) did allow some

Figure 18.

UV irradiation of cycloheximide treated spores prior to cycloheximide removal. Spores suspended in cycloheximide (200 ug/ml) were irradiated with  $250 \text{ J/m}^2$  fluence at the time indicated by the arrow. Cycloheximide was removed via centrifugation, immediately after the application of UV light. The vertical line indicates the time of cycloheximide removal.

Symbols: ( ○ ) percent swelling of control spores  
( □ ) percent swelling of cycloheximide treated spores  
( △ ) percent swelling of cycloheximide treated spores that were exposed to UV light  
( ● ) percent emergence of myxamoebae from control spores  
( ■ ) percent emergence of myxamoebae from cycloheximide treated spores (non-irradiated)  
( ▲ ) percent emergence of myxamoebae from cycloheximide treated spores (irradiated)

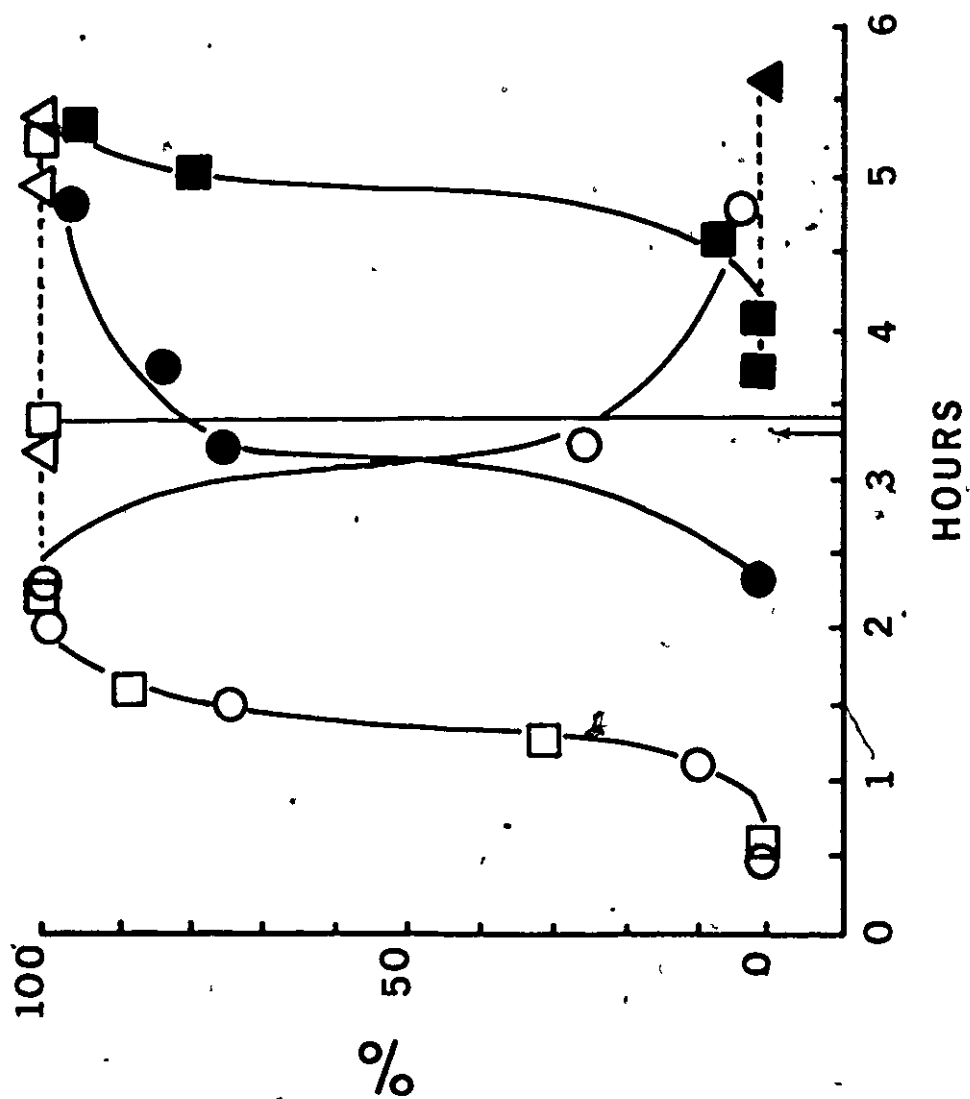





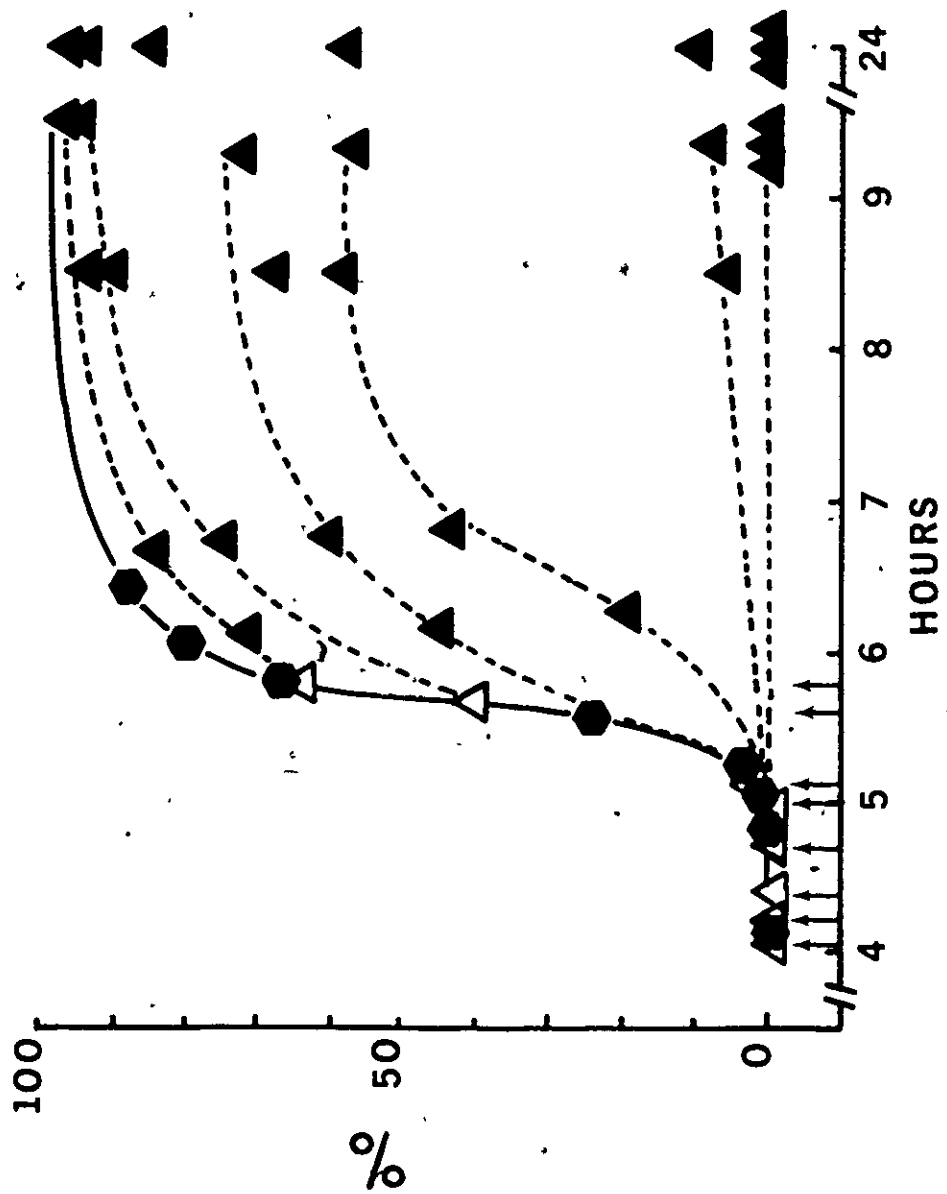
Figure 19a.

UV irradiation of spores after release from cycloheximide inhibition; kinetics of myxamoebae emergence. Heat activated spores were incubated in the presence of cycloheximide for 4 hours at which time emergence in non-treated controls was almost completed (initial germination kinetics shown in Fig. 19b). The cycloheximide (200 ug/ml) was removed via centrifugation at 4 hours after heat activation. The arrows indicate the application of UV light ( $250 \text{ J/m}^2$ ) to eight separate aliquots (2 ml volumes) of washed spore suspension.

Symbols: (  ) percent emergence of myxamoebae from spores after cycloheximide release (non-irradiated)

(  ) percent myxamoebae emergence at the time of UV application

(  ) percent emergence of myxamoebae following UV application (dashed lines)

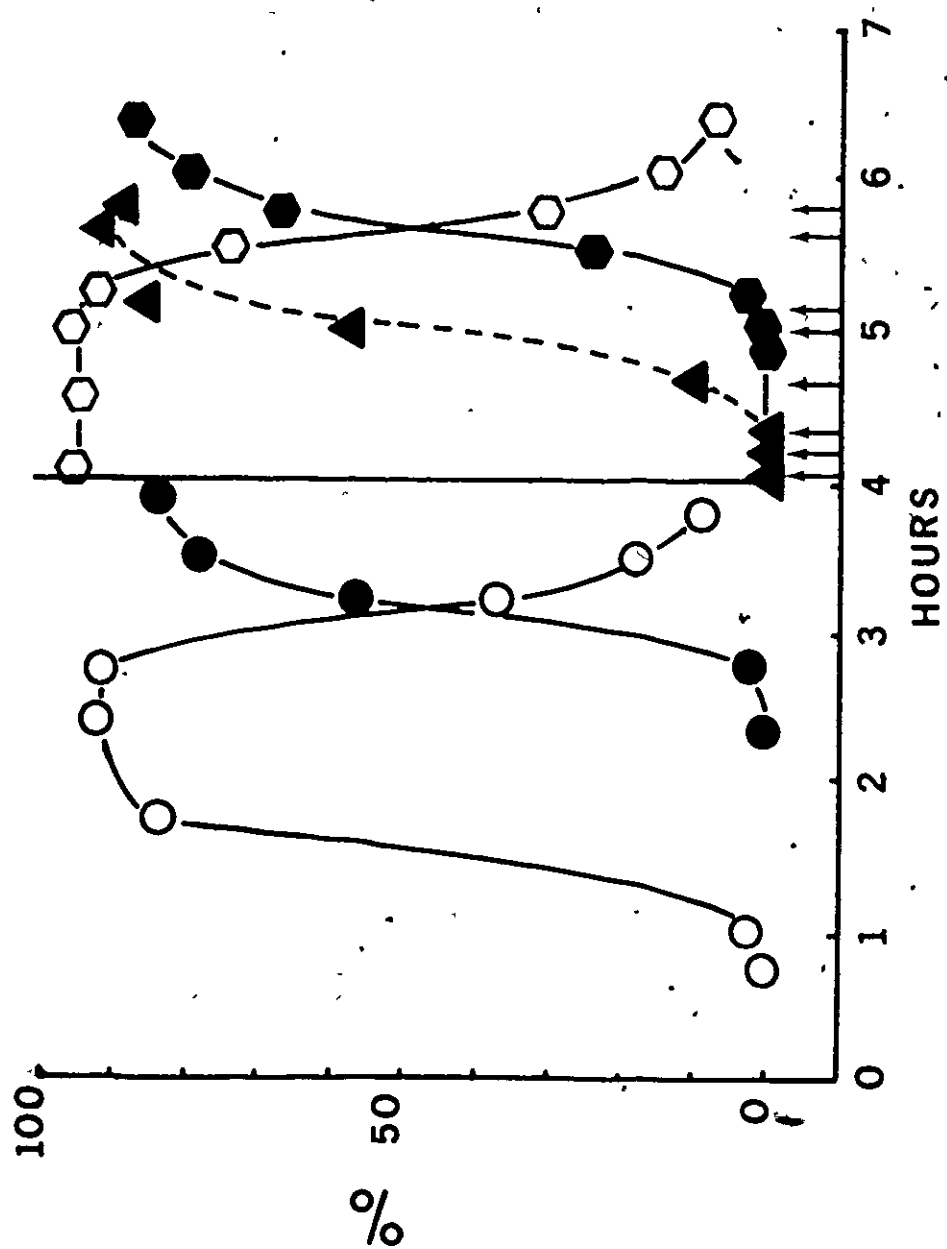


emergence. The kinetics of this emergence is similar to that previously observed for spores irradiated after the competence point in Part I of the Results of this thesis (Fig. 9a). After an initial continued increase in the numbers of emerged myxamoebae, emergence gradually levels off, although somewhat sooner in this particular experiment. Apparently the greater the synchrony of emergence, the less time required for a levelling off in emergence to be observed. From these data it is possible that spores which are released from cycloheximide inhibition, also exhibit a competence point, after which UV light is no longer inhibitory to emergence. This competence point is never achieved in the presence of cycloheximide and in fact occurs at a critical point which occurs some time after the removal of cycloheximide. In Part I, Fig. 9c, a minimal estimate for the competence point was obtained with data values taken at 6 hours after activation. In this experiment, it can be seen that the data values for percent emergence at 24 hours, are only slightly higher than those recorded earlier, and may thus represent a maximal estimate for the competence point. The final values for percent emergence in Fig. 19a (24 hours after activation) are replotted in Fig. 19b at the time of UV application. The kinetics of the normal heat induced activation are also presented in Fig. 19b for comparative purposes. The vertical line (at 4 hours) indicates the time of cycloheximide removal. Spores in which cycloheximide was not removed (open hexagons) remained

Figure 19b.

The final percent emergence of myxamoebae from spores irradiated after release from cycloheximide inhibition. A portion of the data of Fig. 19a was replotted such that the final percent emergence (by 24 hours) is shown at the time of application of UV light ( $250 \text{ J/m}^2$ ). The vertical line indicates the time of cycloheximide removal. The arrows indicate the time of application of UV light to eight separate aliquots of spore suspension after cycloheximide was removed (as in Fig. 19a).

- Symbols:
- ( ○ ) percent swelling of control spores
  - ( ● ) percent emergence of myxamoebae from control spores
  - ( ◐ ) percent emergence of myxamoebae after cycloheximide release (non-irradiated spores)
  - ( ▲ ) final percent emergence of myxamoebae (at 24 hours) from spores irradiated after cycloheximide release
  - ( ◑ ) percent swelling of cycloheximide inhibited spores









maximally swollen throughout the experiment. The arrows in Fig. 19b indicate the time of application of  $250 \text{ J/m}^2$  of UV as in Fig. 19a. When the data is plotted in this manner, the resulting curve (dashed line) is parallel to the initial emergence curve and is sigmoidal in nature. The data of Fig. 19a can also be replotted on a probability scale (as in Fig. 9c, part I). The estimate of the competence point for cycloheximide released spores yields a value which is comparable to that previously estimated for germinating spores after heat activation (Fig. 19c). In Fig. 19c the inverted open triangles represent data values which were obtained within 2 hours after cycloheximide removal (1 hour after commencement of emergence). These values are somewhat lower than those observed at 3 hours after cycloheximide removal (2 hours after commencement of emergence) and later. These early data values (inverted triangles) fall in the range of the minimal estimate for the competence point (about 30 minutes prior to emergence). A maximal estimate for the competence point in this experiment appears to be about 39-40 minutes prior to emergence (closed triangles).

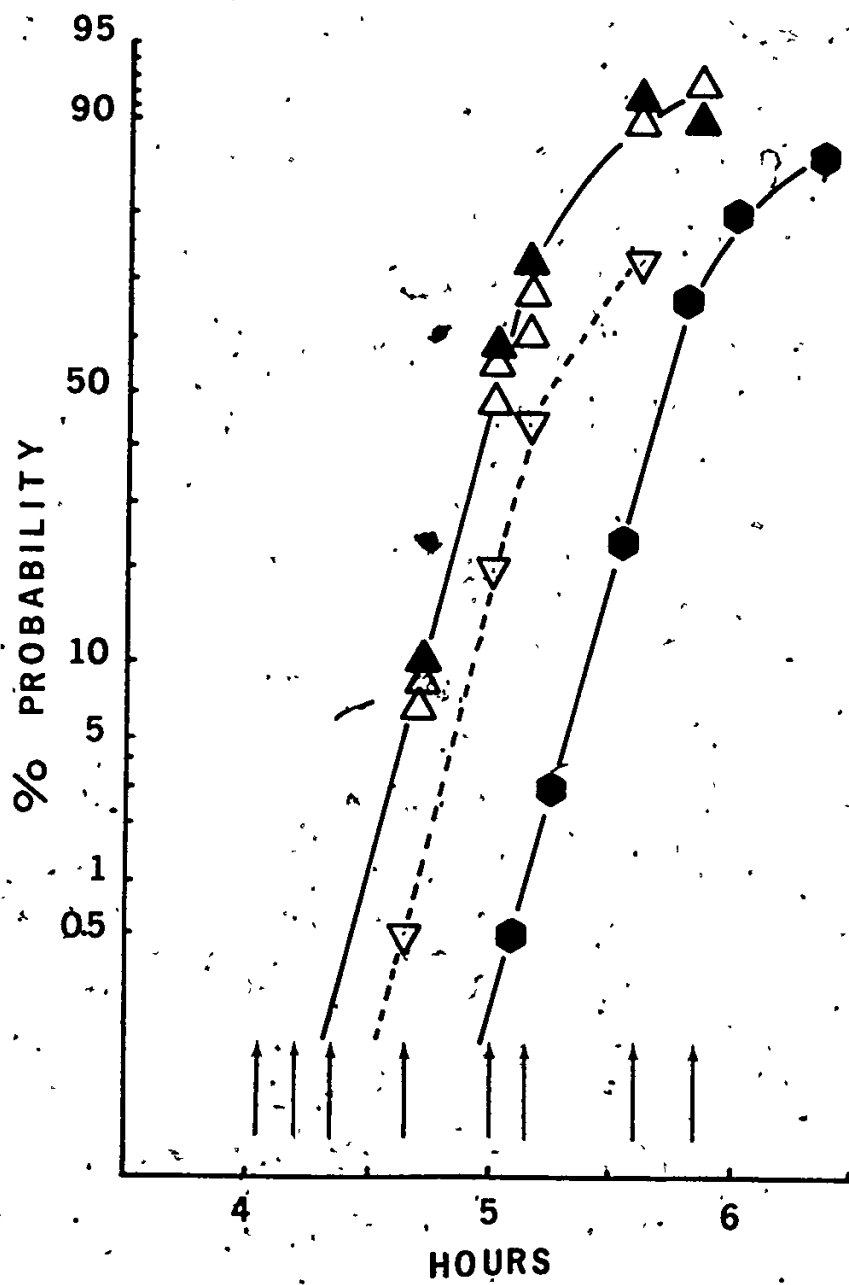
#### 4. Incorporation of radiolabelled leucine

The incorporation of ( $^3\text{H}$ )-leucine (40 Ci/mmmole, NEN) into TCA insoluble precipitates appears to begin sometime during the early swelling phase of germination and increases rapidly just prior to emergence. The accumulation of radiolabelled leucine is inhibited in spores incubated in the

Figure 19c.

The final percent emergence of myxamoebae from spores irradiated after release from cycloheximide inhibition, expressed as a probability function. A portion of the data of Fig. 19a was replotted on a probability scale versus time. The spores were irradiated as described in Fig. 19a and Fig. 19b.

- Symbols: (  ) percent emergence of myxamoebae from control spores after cycloheximide release
- (  ) percent emergence of myxamoebae from irradiated spores at 2 hours following cycloheximide removal
- (  ) percent emergence of myxamoebae from irradiated spores at 3, 4 and 5 hours after cycloheximide removal
- (  ) percent emergence of myxamoebae from irradiated spores at 24 hours after removal of cycloheximide



presence of cycloheximide (200 ug/ml) (Fig. 20). This has previously been observed by a number of researchers (Bacon and Sussman, 1973; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977, 1978). Spores exposed to UV irradiation ( $250 \text{ J/m}^2$ ) immediately after heat activation also did not exhibit the normal accumulation of radiolabelled leucine (Fig. 20).

The total accumulation of leucine label as indicated by phosphate buffer washes (representative of total uptake into the cells) is also inhibited in UV irradiated spores in a manner similar to that of incorporation (Table 3). As already noted it is not believed that the inhibition of uptake is an effect of UV on the transport mechanisms of the cells since cycloheximide inhibited spores exhibited comparable lowered levels of both uptake and incorporation (Table 3).

Since the incorporation and uptake of radiolabelled leucine into spores are comparatively similar as was previously observed for uracil incorporation and uptake, it appears that as previously concluded, the label is immediately incorporated once it enters into the cell. The fact that no uptake was observed in swollen spores that were UV irradiated or treated with cycloheximide, is not totally understood at present. It may be as previously suggested, that swollen spores are permeable to label, but have no mechanisms to retain the label that is not immediately incorporated (Lee, 1972).

Figure 20.

The effect of UV light and cycloheximide on the incorporation of radiolabelled leucine during spore germination. The data presented is an average of two experiments. Spores were irradiated ( $250 \text{ J/m}^2$ ) or suspended in  $200 \text{ ug/ml}$  of cycloheximide immediately after heat activation. The protein precursor ( $^3\text{H}$ )-leucine ( $40 \text{ Ci/mmole}$ , NEN), was used at  $5 \text{ uCi/ml}$ .

Symbols: (  $\bigcirc$  ) percent swelling of control spores  
(  $\bullet$  ) percent emergence of myxamoebae from control spores  
(  $\square$  ) incorporation into control spores  
(  $\hexagon$  ) incorporation into cycloheximide treated spores  
(  $\triangle$  ) incorporation into UV irradiated spores

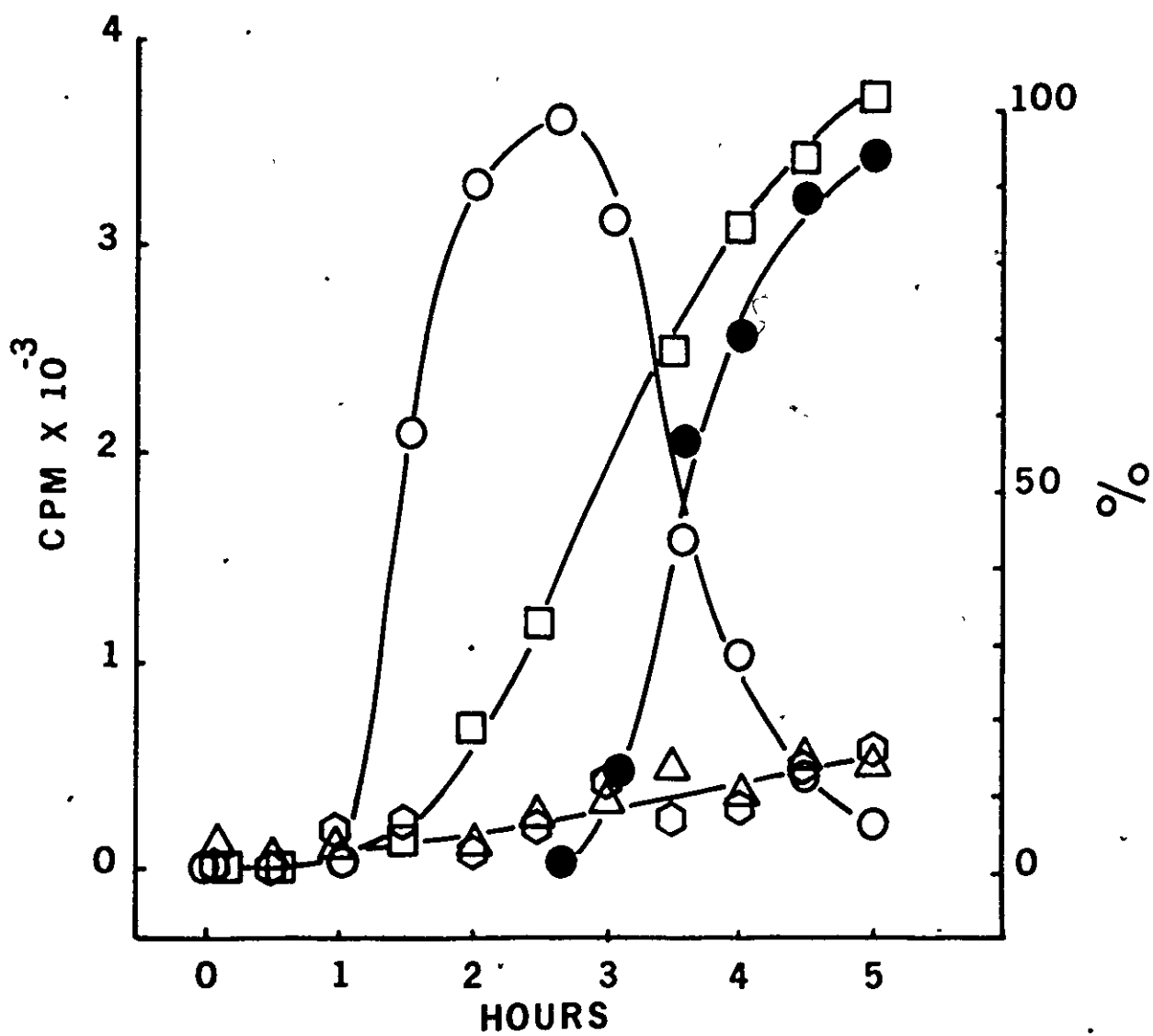


Table 3

Uptake and Incorporation of ( $^{14}\text{C}$ )-leucine during spore  
germination of Dictyostelium discoideum

Time (hrs) after heat activation	CPM per $1 \times 10^7$ spores					
	Uptake			Incorporation		
	control	cyclo.	UV	control	cyclo.	UV
0	516.3	326.9	374.0	177.3	173.4	198.3
5	1731.3	392.4	360.8	1249.1	336.6	435.2

The data for uptake and incorporation was obtained from 2 independent experiments. The precursor ( $^{14}\text{C}$ )-leucine, 290mCi/mM, ICN) was used at 1 uCi/ml. No corrections were made for non-specific binding.

Part III    Ultraviolet Light-induced Inhibition of Enzyme  
Expression During Spore Germination of  
Dictyostelium discoideum

1.    Extraction of total protein from germinating spores

The enzymes B-glucosidase, alkaline phosphatase and trehalase have all been observed to accumulate late in the spore germination sequence (Cotter and Raper, 1970; Tisa and Cotter, 1979). It has been shown by these researchers that the expression of the three enzymes requires protein synthesis. For this reason it would appear that the examination of the expression of these "marker" enzymes could provide a useful means with which to monitor protein synthesis in UV irradiated spores. However, it is important to determine if the protein concentration is constant in any system in which enzyme specific activity is to be used as a measure of protein synthesis. If, for example, much of the total protein is lost during the germination process, then the apparent increase in enzyme specific activities may only be due to the overall decreasing enzyme background (Gustafson and Wright, 1972) or be a result of differential decrease in proteins other than B-glucosidase, alkaline phosphatase and trehalase. It is also important to consider whether the method of protein extraction results in values which are representative of the total protein in the different cell types of the organism. The total protein extracted from the three different cell types (dormant spores, swollen spores and emerged myxamoebae) was compared (Table 4). Also, the



Table 4 Extraction of Total Protein During Spore Germination

Percent Cells Cell	100% Dormant Spores	96% Swollen Spores	86% Emerged Myxamoebae
Concentration per ml x 10 <sup>7</sup>	1.18 2.35 4.70	1.20 2.39 4.78	1.13 2.35 4.50
Number of Cells Disrupted per ml x 10 <sup>7</sup>	1.15 2.33 4.67	1.19 2.39 4.77	1.12 2.25 4.49
Number of Intact Cells per ml x 10 <sup>5</sup>	2.05 2.10 3.35	0.27 0.55 0.90	0.14 0.12 0.17
Percent			
Efficiency of Cell Breakage	98.26 99.11 99.29	99.81 99.80 99.84	99.88 99.95 99.96
Average	98.89	99.82	99.93
mg protein per sample	0.170 0.195 0.390	0.126 0.213 0.391	0.127 0.221 0.392
mg protein per 1 x 10 <sup>7</sup> cells	0.147 0.083 0.084	0.105 0.089 0.082	0.112 0.098 0.087
average mg protein per 1 x 10 <sup>7</sup> cells	0.105	0.092	0.099

All cells in 5 ml volumes were disrupted by 3 passages through a French Pressure Cell at 20,000 lb/sq. in.

The determination of mg protein per sample is an average of two assays by the method of Bradford using albumin as the protein standard.

relative effectiveness of cellular disruption of the three cell types at various cell concentrations was compared (Table 4). Dormant spores are only slightly more resistant to disruption than swollen spores or emerged myxamoebae. The higher concentrations did increase the efficiency of breakage for all three cell types. In all cases the efficiency of breakage with three passages through a French pressure cell at 20,000 lb./sq. in. was better than 98% as estimated by hemocytometer counts of intact cells which remained in the crude lysate. The lysates from the disrupted cells were centrifuged as previously described and assayed for total protein by the method of Bradford using albumin as the protein standard (see Materials and Methods). It was observed that dormant spores, swollen spores and emerged myxamoebae all yielded the same relative amount of protein. Although this method does not allow an estimation of any protein turnover that may occur, as would a more sensitive radiolabelling technique (Wright and Anderson, 1960a, b), it is sufficient to assure that the total protein content is not changing significantly enough to influence the changes in enzyme specific activity which occur during spore germination. It also appears that the French pressure cell method of cellular disruption is equally effective for the extraction of total protein from all three cell types.

## 2. B-glucosidase expression

The increase in specific activity of the enzyme B-glucosidase appears to occur concomitantly with the emergence

of myxamoebae (Fig. 21). This has been shown previously (Tisa and Cotter, 1980; Chan and Cotter, submitted). It has also been demonstrated by Chan and Cotter (submitted) that B-glucosidase occurs in two forms in spores of Dictyostelium discoideum. B-glucosidase form II is present in the dormant spore and its activity decreases during heat-induced activation. It has been observed that the increase in B-glucosidase expression that occurs concomitant with emergence is due to the synthesis B-glucosidase form I (Chan and Cotter, submitted). The accumulation of B-glucosidase (form I) is inhibited in spores treated with UV irradiation. In Fig. 21 the enzyme specific activity at the time of UV irradiation is indicated by the open square, and the enzyme specific activity of irradiated spores which were crushed and assayed at various time intervals after UV treatment is represented by the closed squares. Note that the increase in enzyme specific activity stops abruptly (less than 15 minutes). The abrupt enzyme inhibition shortly after UV is not due to simple enzyme inactivation since irradiated and non-irradiated supernatants of 5 hour germinated spores exhibited no significant difference when assayed for B-glucosidase specific activity (Table 5). In the same experiment the percent emergence at the time of UV (indicated by the open triangle) continues to increase for some time after irradiation and then levels off (as indicated by the closed triangles). In more complete experiments (Fig. 22a) spore suspensions were irradiated at a number of time intervals during germination, as indicated by the arrows,

Figure 21.

The effect of a single irradiation treatment upon the expression of B-glucosidase specific activity and myxamoebae emergence. The data presented is from 1 independent experiment. The arrow indicates the time of application of  $250 \text{ J/m}^2$  of UV light to a large volume of germinating spores.

- Symbols: (■) time of heat activation
- (○) percent swelling of control spores
- (●) percent emergence of myxamoebae from control spores
- (△) percent emergence of myxamoebae from control spores at the time of irradiation
- (▲) percent emergence of myxamoebae from irradiated spores
- (⬡) enzyme specific activity of control spores
- (□) enzyme specific activity of control spores at the time of irradiation
- (■) enzyme specific activity of irradiated spores

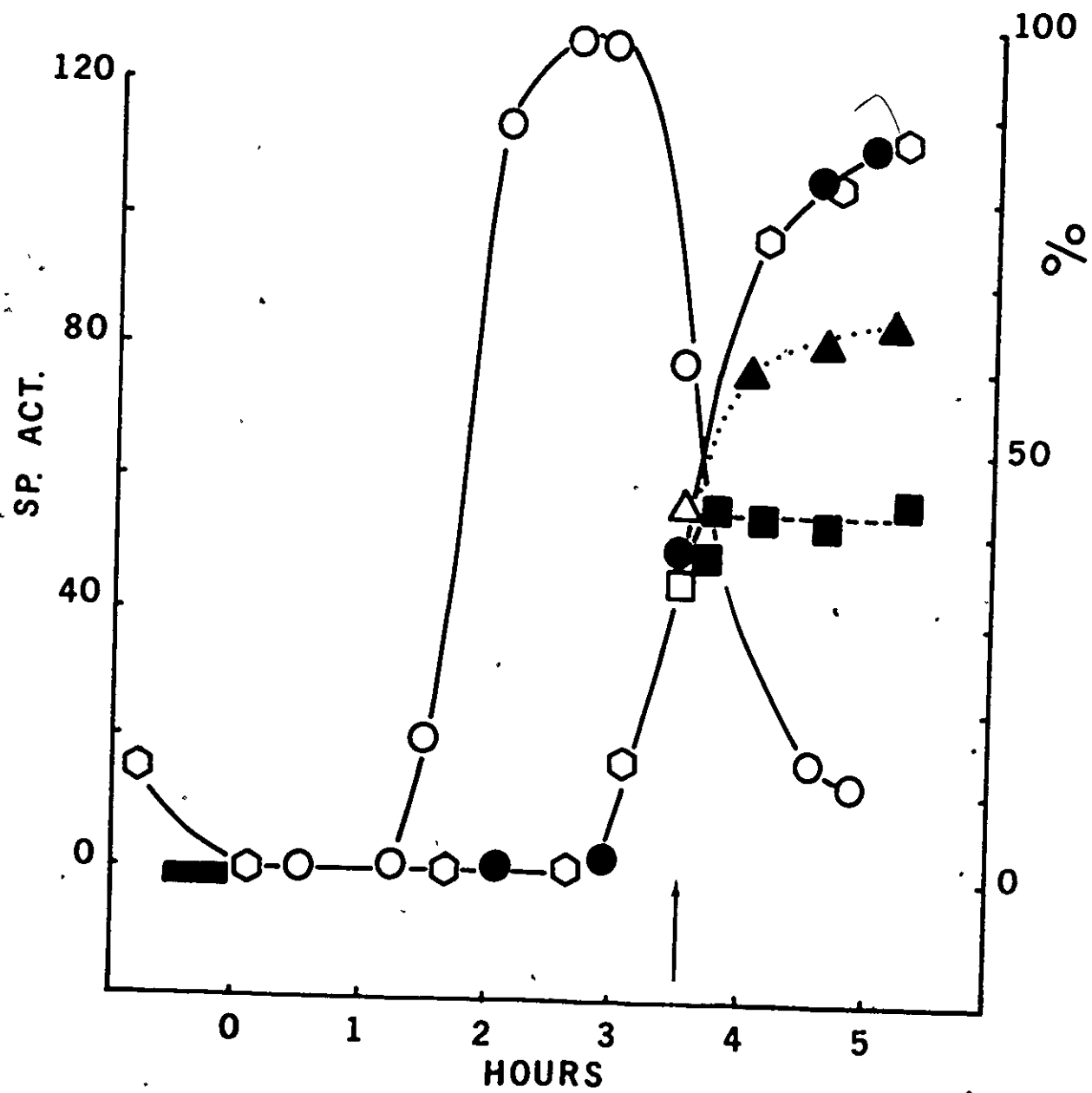


Figure 22a.

The effect of irradiation treatments during spore germination upon the expression of B-glucosidase specific activity and myxamoebae emergence. The data presented is an average of two assays; the arrows indicate the time of application of UV at a fluence of  $250 \text{ J/m}^2$  to 8 separate aliquots from a flask of germinating spores. These aliquots were incubated separately and the number of emerged myxamoebae as well as the enzyme specific activity of the samples were determined after germination had been completed in control spores (5.5 to 6 hours after activation).

- Symbols: (■) time of heat activation
- (●) percent emergence of myxamoebae from control spores
- (△) percent emergence of myxamoebae from control spores at the time of irradiation
- (▲) percent emergence of myxamoebae from irradiated spores
- (⬡) enzyme specific activity of control spores
- (□) enzyme specific activity of control spores at the time of irradiation
- (■) enzyme specific activity of irradiated spores

Note: The dashed and dotted lines are representative extrapolations for B-glucosidase enzyme specific activity and emergence of myxamoebae after UV irradiation, respectively.

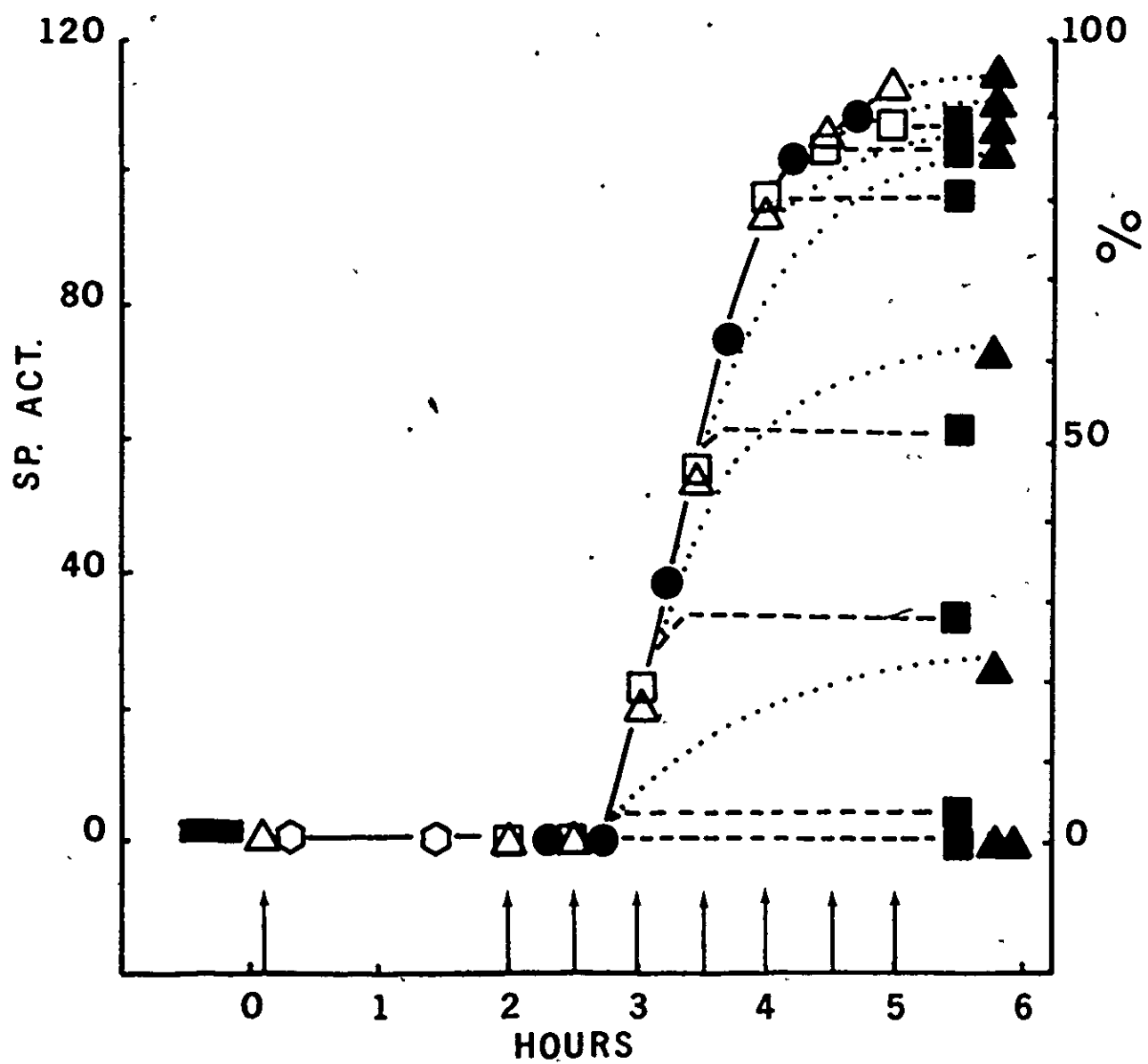


Table 5

## UV Irradiation of Crude Enzyme Preparations

Sample	Exp. No.	B-glucosidase Specific Activity	Trehalase Specific Activity	Alkaline Phosphatase Specific Activity
Control	1	114.8	13.4	35.5
	2	106.9	18.2	50.6
UV Irradiated	1	103.4	13.9	36.8
	2	108.7	18.5	48.5

The supernatants of crude cell lysates were irradiated in 2 ml volumes in 5 cm diameter glass petri dishes that were held on ice. Control supernatants received the same treatment except the petri dish glass covers were not removed during the irradiation, to prevent the penetration of 254 nm UV light. All enzyme assays were performed as previously described in the Materials and Methods, and all enzyme specific activities are expressed as nmoles/min per mg protein for comparative purposes. The trehalase specific activity may be divided by a factor of 33.33 to convert the values from nmoles/min/mg to umoles/30 min/mg as expressed in Fig. 24 - 27.

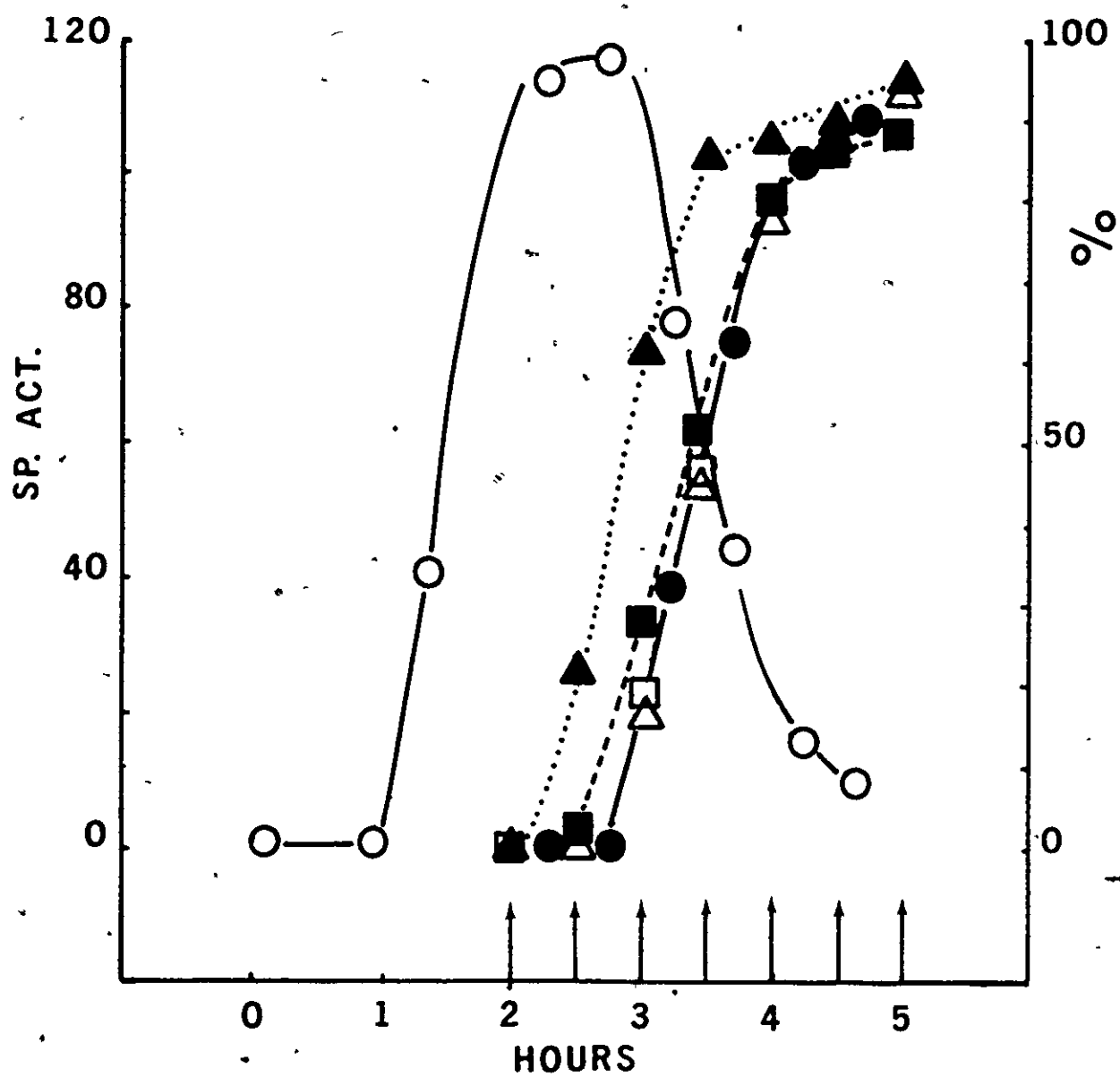


and the suspensions were incubated in separate tubes. The final percent emergence and enzyme specific activity were determined for the separate applications of UV 6 hours after activation. UV irradiation at any time prior to maximum spore swelling (up to 2 hours in this experiment) completely inhibited the increase in enzyme expression as well as the emergence of myxamoebae. The kinetics of emergence and enzyme expression following UV application at various time intervals after maximum swelling is represented by the dashed and dotted lines respectively (Fig. 22a). Irradiated dormant spores exhibited similar enzyme specific activity after 5 hours of incubation as did non-irradiated dormant spores (data not shown). It is observed that a certain fraction of the population of germinating spores is capable of emerging even after UV application and the cessation of  $\beta$ -glucosidase accumulation. The data from Fig. 22a can be replotted such that the final values for percent emergence and enzyme specific activity are shown at the time of UV application (Fig. 22b). In this case the curves generated are also sigmoidal in nature and parallel to the initial emergence and enzyme specific activity curves. However, the final percent emergence (6 hours after activation) results in a sigmoidal curve which appears considerably earlier than the curve obtained for enzyme expression. From the data plotted in Fig. 22b one may make a minimal estimate (about 30 minutes) for the competence point for emergence which is in agreement with previously observed values (Fig. 19b, 19c).

Figure 22b.

The final percent emergence of myxamoebae and the final B-glucosidase specific activity of spores irradiated during germination. A portion of the data from Fig. 22a was replotted such that the final values (6 hours after activation) for percent emergence and enzyme specific activity are shown at the time of UV application (as indicated by the arrows).

- Symbols:
- ( ○ ) percent swelling of control spores
  - ( ● ) percent emergence of myxamoebae from control spores
  - ( △ ) percent emergence of myxamoebae from control spores at the time of irradiation
  - ( ▲ ) the final percent emergence of myxamoebae from irradiated spores 6 hours after activation
  - ( □ ) enzyme specific activity of control spores at the time of irradiation
  - ( ■ ) the final enzyme specific activity of irradiated spores 6 hours after activation



In addition the expression of B-glucosidase occurs after the competence point has already been attained.

### 3. Alkaline phosphatase expression

The increase in specific activity of alkaline phosphatase began somewhat earlier than B-glucosidase or trehalase, i.e., during the swelling phase of germination (Tisa and Cotter, 1979). This early expression of a low level of alkaline phosphatase specific activity was not inhibited by UV since spores which were irradiated immediately after activation and any time prior to maximum swelling (about 2 1/2 hours in this experiment) developed the same enzyme activity as maximally swollen control spores (Table 6). This value was only about 2 fold higher than the zero hour control value for alkaline phosphatase specific activity (Table 6). Irradiated dormant spores exhibited similar enzyme specific activity as non-irradiated dormant spores which was only slightly higher than the basal level of enzyme activity observed in freshly activated spores.

In Fig. 23a the open squares represent the enzyme activity values at the time of UV application and the closed squares represent the enzyme specific activities of irradiated spores 6 hours after heat activation. The values observed at 6 hours were in most cases only slightly higher from those observed at the time of irradiation. The kinetics of the inhibition of alkaline phosphatase expression thus appeared to be similar to that of B-glucosidase expression and are

Table 6

Expression of Alkaline Phosphatase Specific Activity in  
Spores Irradiated Prior to Maximum Spore Swelling

Time of UV Treatment (Hours after Activation)	Alkaline phosphatase specific activity	
	At Time of UV	6 hours after activation
Dormant*	8.57*	8.15*
0	6.58	13.39
0.5	6.41	11.98
1.0	6.57	11.10
1.5	6.89	11.51
2.0	8.90	12.57

\* non activated controls

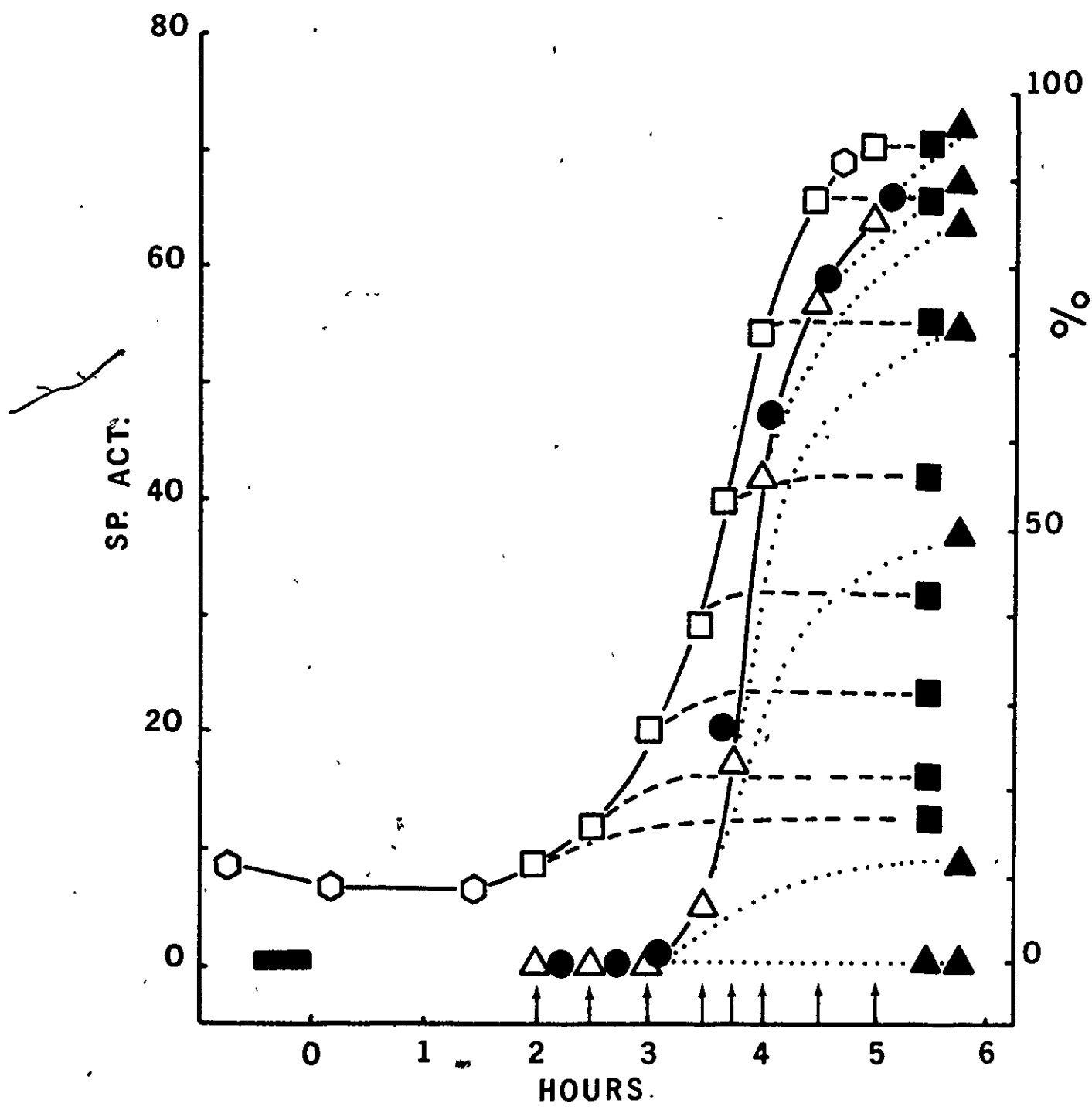
The data presented was averaged from 4 independent experiments. Spores were irradiated ( $250 \text{ J/m}^2$ ) at the times indicated; control spores (non-irradiated) were assayed for enzyme activity at the indicated times after activation. Irradiated spores were incubated separately and assayed for enzyme activity at 6 hours after activation. Alkaline phosphatase specific activity was determined as described in the Materials and Methods section.

Figure 23a.

The effect of irradiation during spore germination upon the expression of alkaline phosphatase specific activity and myxamoebae emergence. The data presented is averaged from four independent experiments. Arrows indicate the time of 8 separate applications of UV at a fluence of  $250 \text{ J/m}^2$ .

- Symbols: (■) time of heat activation
- (●) percent emergence of myxamoebae from control spores
  - (△) percent emergence of myxamoebae from control spores at the time of irradiation
  - (▲) percent emergence of myxamoebae from irradiated spores 6 hours after activation
  - (○) control enzyme specific activity
  - (□) enzyme specific activity of control spores at the time of irradiation
  - (■) enzyme specific activity of irradiated spores at 6 hours after activation.

Note: The dashed and dotted lines are representative extrapolations for alkaline phosphatase enzyme specific activity and emergence of myxamoebae after UV irradiation, respectively.



represented as such by the extrapolated dashed lines. Again, the specific activities of irradiated and non-irradiated 5 hour control supernatants were not significantly different (Table 5). The open and closed triangles of Fig. 23a represent the percent emergence at the time of irradiation, and the percent emergence of myxamoebae from irradiated spores 6 hours after activation, respectively. It is again observed that amoebae are capable of emerging for some time after UV irradiation, even though alkaline phosphatase accumulation has ceased.

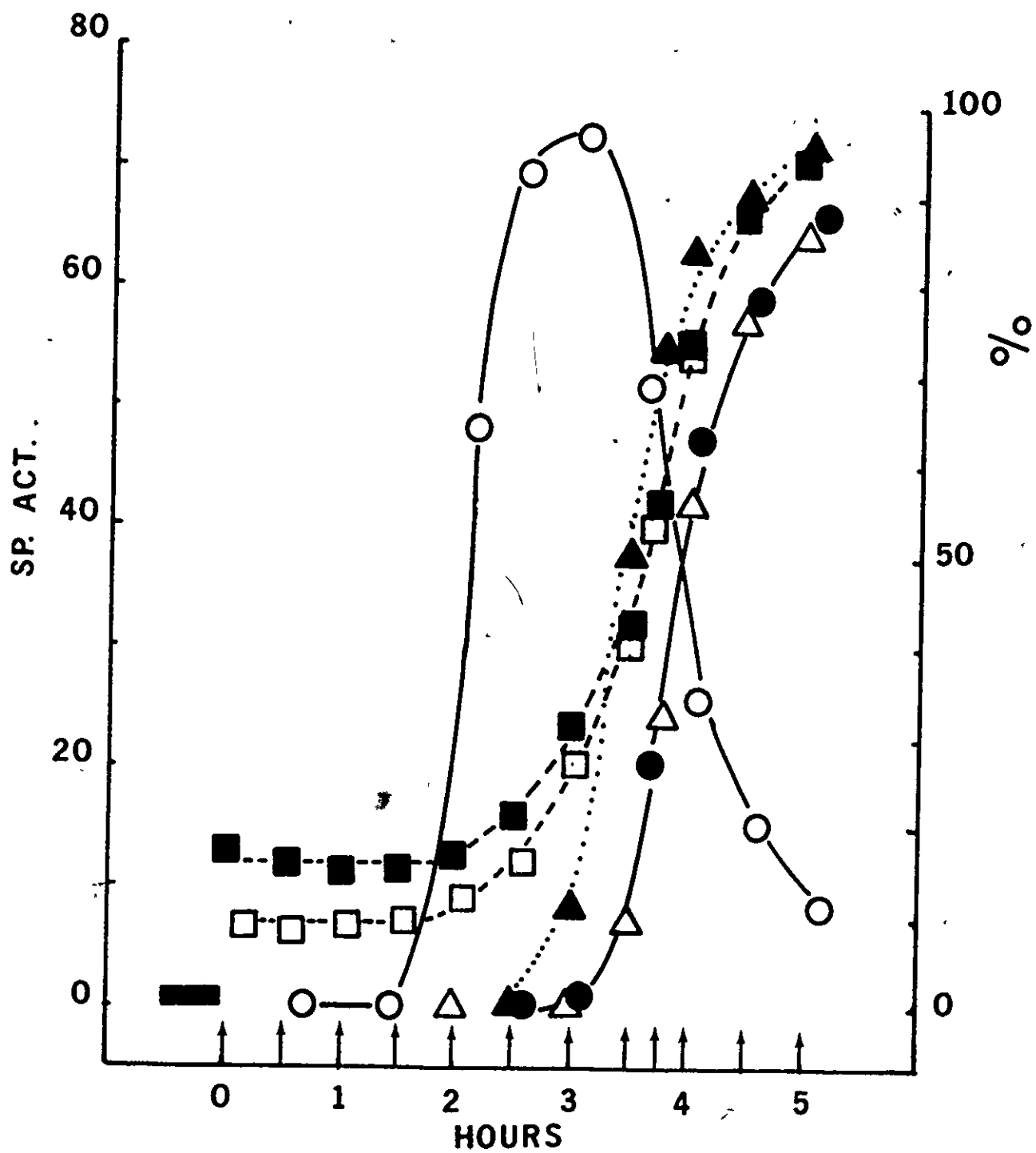
A portion of the data from Fig. 23a can be replotted such that the final values for percent emergence and enzyme specific activity at 6 hours are shown at the time of UV application (Fig. 23b). When the data are presented in this manner, the curve generated for final percent emergence 6 hours after UV (closed triangles) is sigmoidal in nature and relatively parallel to the initial emergence curve (closed circle). An estimation of the competence point which can be obtained by comparing the temporal separation of the two sigmoidal curves yields a value which is in agreement with previously established minimal estimates for competence (Fig. 9c, Part I). Though there does seem to be a small amount of alkaline phosphatase expression which occurs early (perhaps prior to the competence point) (Fig. 23b), the bulk of alkaline phosphatase specific activity is expressed late (after the competence point) as was observed for B-glucosidase (Fig. 22b).



Figure 23b.

The final percent emergence of myxamoebae and the final alkaline phosphatase specific activity of spores irradiated during germination. A portion of the data from Fig. 23a was replotted, such that the final values (6 hours after activation) for percent emergence (dotted line) and enzyme specific activity (dashed line) are shown at the time of UV application ( $250 \text{ J/m}^2$ ) as indicated by the arrows.

- Symbols: (■) time of heat activation
- (○) percent swelling of control spores
- (●) percent emergence of myxamoebae from control spores
- (△) percent emergence of myxamoebae from control spores at the time of irradiation
- (▲) final percent emergence of myxamoebae from irradiated spores 6 hours after activation
- (□) enzyme specific activity of control spores at the time of UV
- (■) final enzyme specific activity of irradiated spores 6 hours after activation



#### 4. Trehalase expression

The increase of trehalase enzyme specific activity appears to begin just prior to the emergence of myxamoebae in heat activated spores. This "stage specific" expression of trehalase is inhibited in spores incubated in the presence of cycloheximide (Cotter and Raper, 1970). Spores which are irradiated at any time prior to maximum swelling (about 2 hours) also exhibited no increase in enzyme specific activity (Table 7). Spores which are irradiated at intervals after maximum swelling exhibit increases in trehalase specific activity which are correlated with the time of UV application, as was observed previously for B-glucosidase and alkaline phosphatase enzymes. The effect of UV applied at various times during germination can be seen in Fig. 24; the germination kinetics have been plotted separately (Panel A) from the enzyme kinetics (Panel B) to avoid confusion. In Fig. 24, Panel A, the open triangles indicate the percent emergence at the time of UV and the closed triangles indicate the percent emergence achieved by 6 hours after activation. If the final percent emergence achieved is plotted at the time of UV irradiation, then another sigmoidal curve is generated (inverted closed triangles) which is parallel to the initial emergence curve. As previously observed, the temporal difference between the two curves is approximately 30 minutes. This is in good agreement with the previous minimal estimation for the competence point. In Fig. 24, Panel B, the open squares represent the trehalase specific activity at the

Table 7

Expression of Trehalase Specific Activity in Spores  
Irradiated Prior to Maximum Spore Swelling

Time of UV Treatment  (Hours after Activation)	Trehalase Specific Activity (nmoles/min per mg protein)	
	At Time of UV	6 Hours After Activation
dormant*	1.51*	1.19*
0.5	1.77	0.80
1.0	1.43	1.03
1.5	1.17	0.90
2.0	0.99	0.70

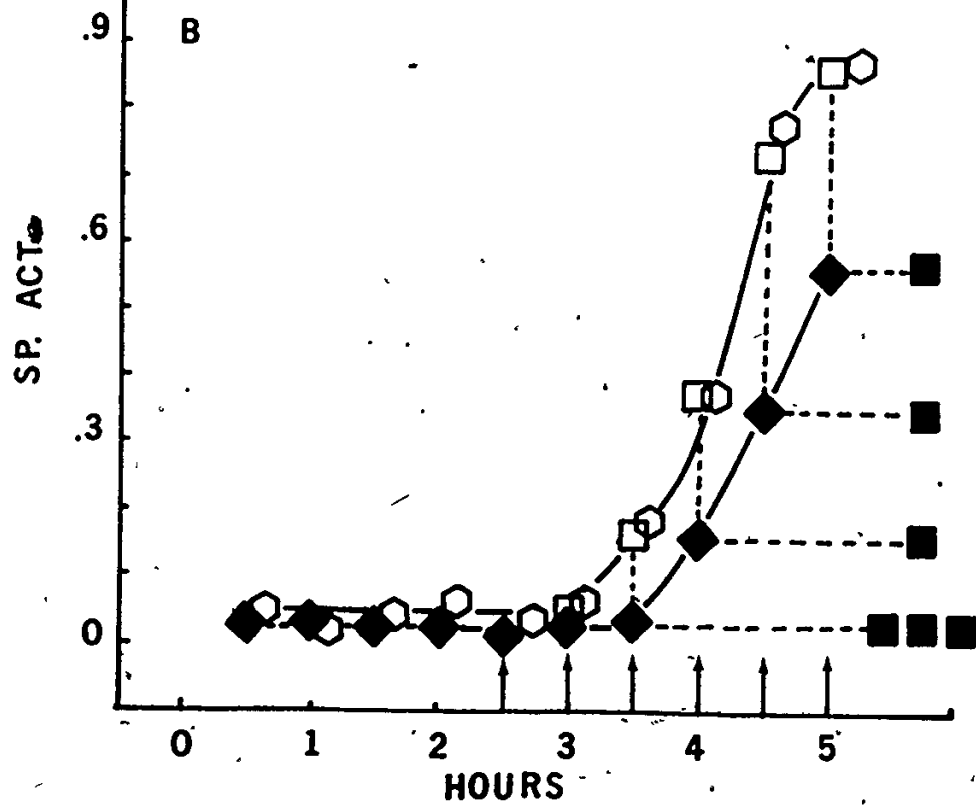
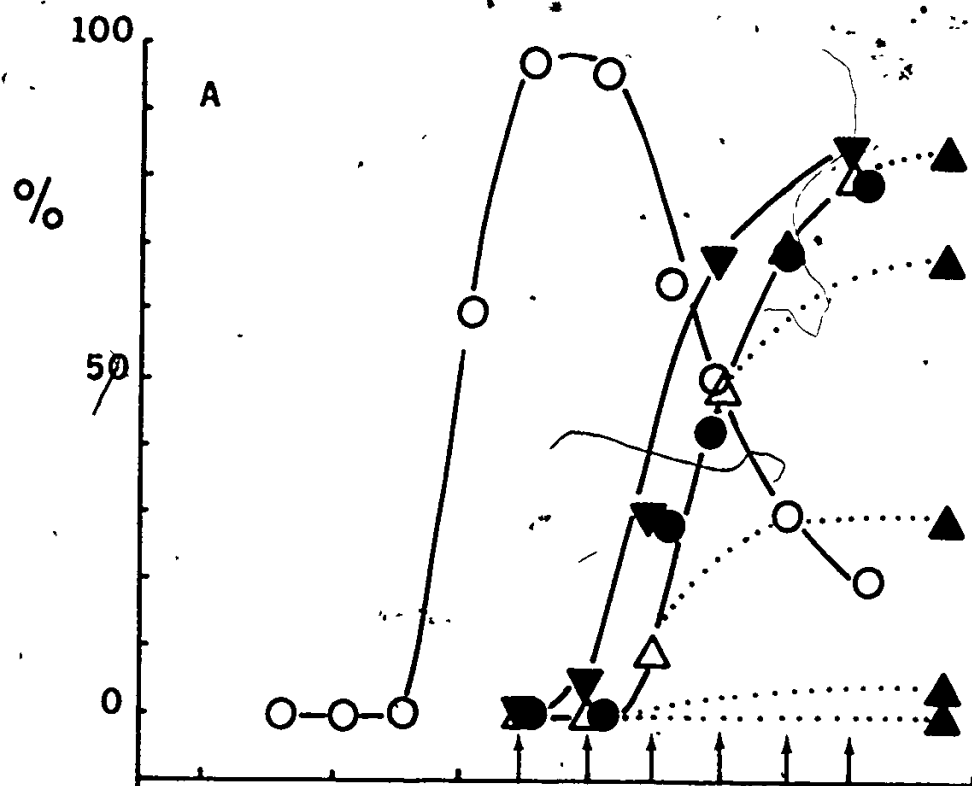
\* Control nonactivated

The data presented is from 1 independent experiment. Spores were irradiated ( $250 \text{ J/m}^2$ ) at the times indicated; control spores (non-irradiated) were assayed for enzyme activity at the indicated times after activation. Irradiated spores were incubated separately and assayed for enzyme activity at 6 hours after activation. Trehalase specific activity was determined as previously described in the Materials and Methods section. (The trehalase specific activity may be converted to  $\mu\text{moles}/30 \text{ min}$  per mg protein as expressed in Fig. 24-27, by dividing by a factor of 33.33).

Figure 24.

The effect of UV irradiation during spore germination upon the expression of trehalase specific activity and myxamoebae emergence. The data presented is an average of three assays from 1 independent experiment. The arrows indicate the time of application of  $250 \text{ J/m}^2$  of UV light to 6 separate aliquots of germinating spores. The emergence kinetics are shown in Panel A. In Panel B, the kinetics of trehalase accumulation in control spores are compared to the final values of trehalase specific activity in irradiated spores observed 6 hours after activation (closed squares). The closed diamonds indicate the enzyme values for irradiated spores at 6 hours plotted against the time of UV application. The dashed lines are extrapolations to illustrate the degree of enzyme decrease from initial enzyme values at the time of UV application (open squares).

- Symbols:
- ( ○ ) percent swelling of control spores
  - ( ○ ) percent emergence of myxamoebae from control spores
  - ( △ ) percent emergence at the time of UV application
  - ( ▲ ) percent emergence of myxamoebae from irradiated spores 6 hours after activation
  - ( ▼ ) percent emergence of myxamoebae from irradiated spores 6 hours after activation, plotted at the time of UV application
  - ( ⬡ ) enzyme specific activity of control spores
  - ( □ ) enzyme specific activity at the time of UV application
  - ( ■ ) enzyme specific activity of irradiated spores 6 hours after activation
  - ( ◆ ) enzyme specific activity of irradiated spores 6 hours after activation, plotted at the time of UV application



time of UV irradiation. The closed squares show the levels of enzyme specific activity observed at about 6 hours after activation. It was noted that the enzyme specific activity of the irradiated spores (which were incubated until 6 hours after activation) was considerably lower than the enzyme values estimated at the time of UV application. The degree of this drop in enzyme level after irradiation is more apparent if the enzyme values observed at 6 hours are plotted at the time of UV application (closed diamonds). Indeed, the final curve for trehalase enzyme expression is shifted temporally even farther to the right of the initial enzyme curve. When the data presented in Panel B are compared with the emergence kinetics presented in Panel A, it is noted that the expression of trehalase specific activity occurs after the competence point has already been reached.

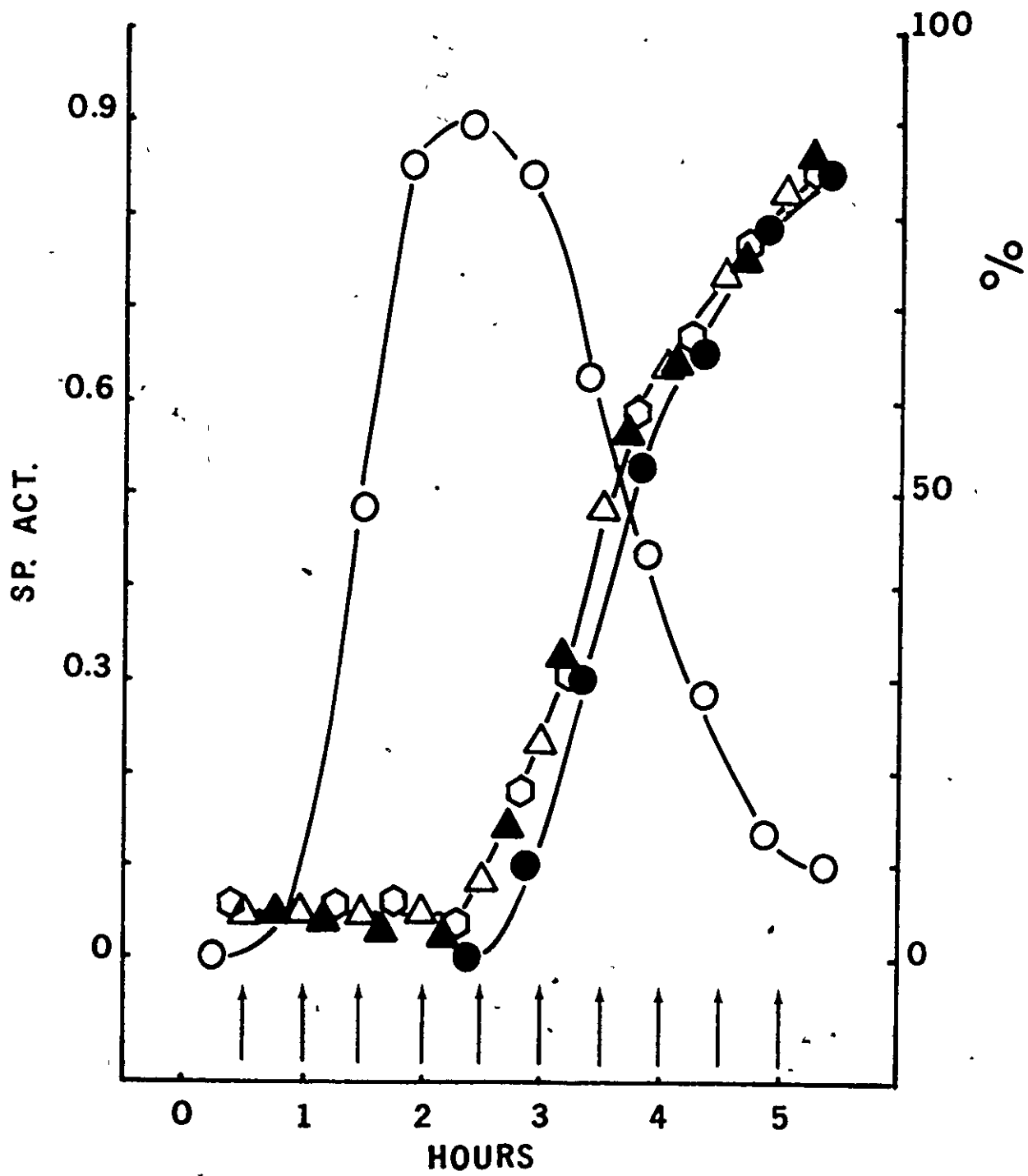
The dramatic decreases in trehalase specific activity are not due to direct UV inactivation of the enzyme since trehalase specific activities of irradiated and non-irradiated control supernatants are comparable (Table 5). Also, if the trehalase specific activity was determined immediately after UV irradiation (no period of incubation prior to cellular disruption) the enzyme levels are comparable to the levels observed for non-irradiated controls (Fig. 25). The data presented in Fig. 25 are from one independent experiment. The open hexagons show the trehalase specific activity of non-irradiated controls; the bulk of enzyme accumulation can be seen to occur about 15-20 minutes prior to emergence. The

Figure 25.

The effect of UV upon the expression of trehalase specific activity immediately after irradiation of germinating spores. The data presented is an average of three assays from 1 independent experiment. The arrows indicate the time of application of  $250 \text{ J/m}^2$  to 10 separate aliquots of germinating spores. Irradiated spores were crushed and assayed for trehalase specific activity immediately after the application of UV light (within 6 - 12 minutes), as described in the Materials and Methods.

Symbols: ( ○ ) percent swelling of control spores  
 ( ● ) percent emergence of myxamoebae from control spores  
 ( ⬡ ) enzyme specific activity of control spores  
 ( △ ) enzyme specific activity at the time of UV application  
 ( ▲ ) enzyme specific activity of irradiated spores immediately after UV application





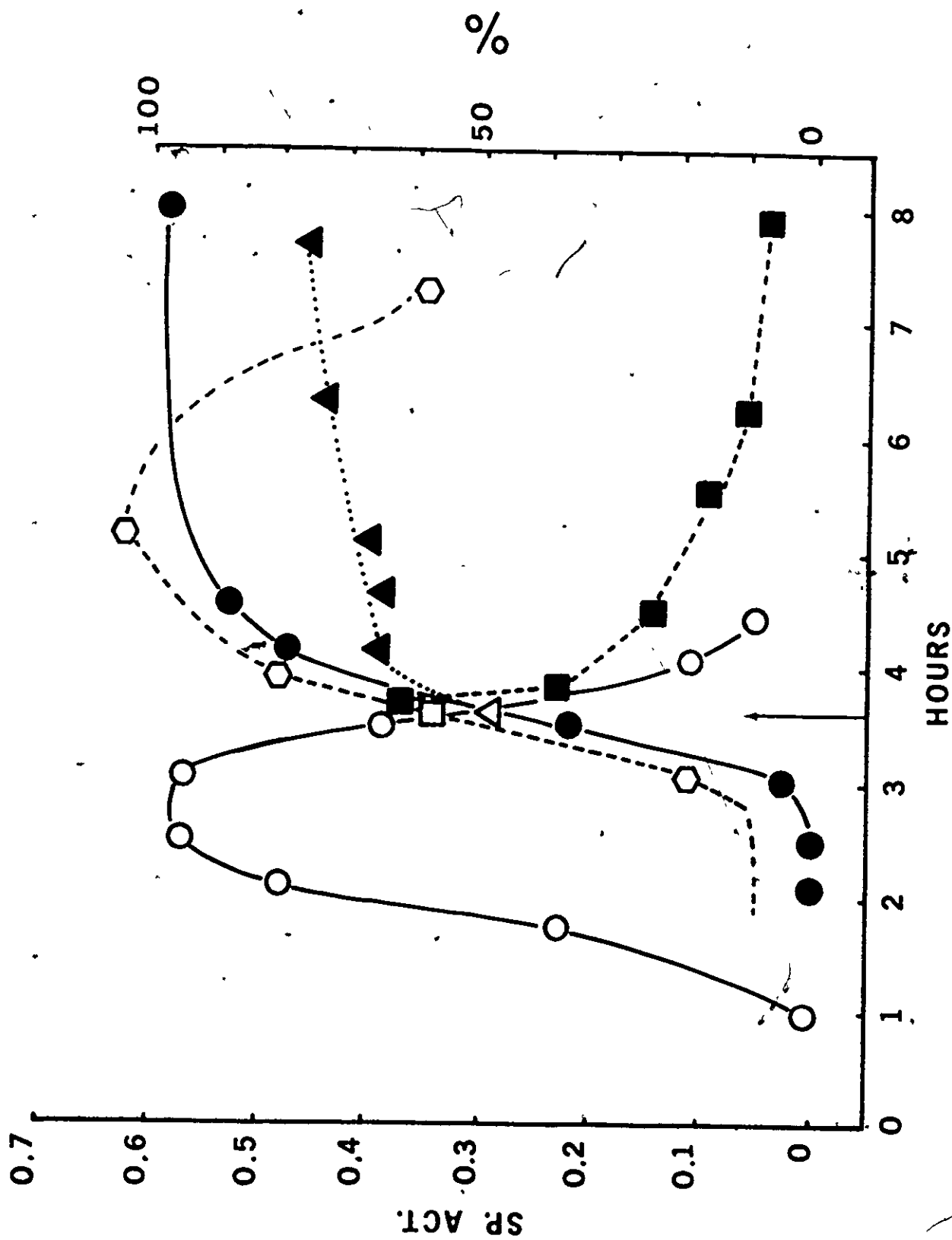
open triangles indicate the enzyme levels at the time of UV irradiation. The closed triangles indicate the enzyme levels of irradiated spores that were crushed and assayed within 6-12 minutes after UV irradiation. The enzyme specific activity of irradiated spores can continue to increase for a very short time following the application of UV light. This has been previously observed for both B-glucosidase and alkaline phosphatase enzymes. The exact duration of continued enzyme accumulation can only be estimated from Fig. 25 and would appear to be less than 15 minutes in time.

The actual kinetics of the drop in trehalase specific activity after UV irradiation are shown in Fig. 26. The data presented is from one independent experiment. A large volume of germinating spores was irradiated ( $250 \text{ J/m}^2$ ) at the time indicated by the arrow and aliquots were assayed for trehalase specific activity at various time intervals after UV irradiation. The numbers of emerged myxamoebae following UV irradiation were also monitored. The data suggest that there is a logarithmic decrease in trehalase specific activity which begins shortly after UV irradiation and then levels off. The trehalase specific activity of non-irradiated spores was also monitored after emergence had been completed. It appears that the trehalase specific activity of non-irradiated controls also drops after a maximum value had been attained at 5 hours after activation (depending on the time that emergence is completed). It has been shown that the drop in trehalase

Figure 26.

Kinetics of loss in trehalase enzyme specific activity after application of UV light. A large volume of germinating spores was irradiated ( $250 \text{ J/m}^2$ ) when emergence had reached about 50 % in control spores, as indicated by the arrow. The data presented is from one independent experiment.

- Symbols:
- ( ○ ) percent swelling of control spores
  - ( ● ) percent emergence of control spores
  - ( △ ) percent emergence at the time of UV application
  - ( ▲ ) percent emergence following UV irradiation
  - ( ⬡ ) enzyme specific activity of control spores
  - ( □ ) enzyme specific activity at the time of UV application
  - ( ■ ) enzyme specific activity following UV irradiation




specific activity of control spores after 5 hours and the drop in enzyme level of UV irradiated spores is paralleled by a concomitant increase in the appearance of trehalase in the medium of incubation (Chan, personal communication). It appears that trehalase enzyme, which is apparently released to the external medium, cannot be detected unless the spores are incubated in sufficiently high concentrations. It has also been shown that the trehalase activity in the external medium decreases with time which is suggestive of an apparent denaturation or degradation of trehalase enzyme after it is released from the amoebae (Chan, personal communication).

The kinetics of trehalase expression after UV irradiation at various times during germination is graphically depicted in Fig. 27. The data presented is from Fig. 25 in which trehalase specific activity was determined immediately after UV irradiation and from Fig. 24 in which the final values of trehalase specific activity (6 hours after activation) were determined. The kinetics of trehalase expression after UV irradiation are markedly different from the kinetics observed previously for B-glucosidase expression (Fig. 22a) and alkaline phosphatase expression (Fig. 23a) after UV irradiation.

##### 5. Inhibition of enzyme expression at minimal UV fluences

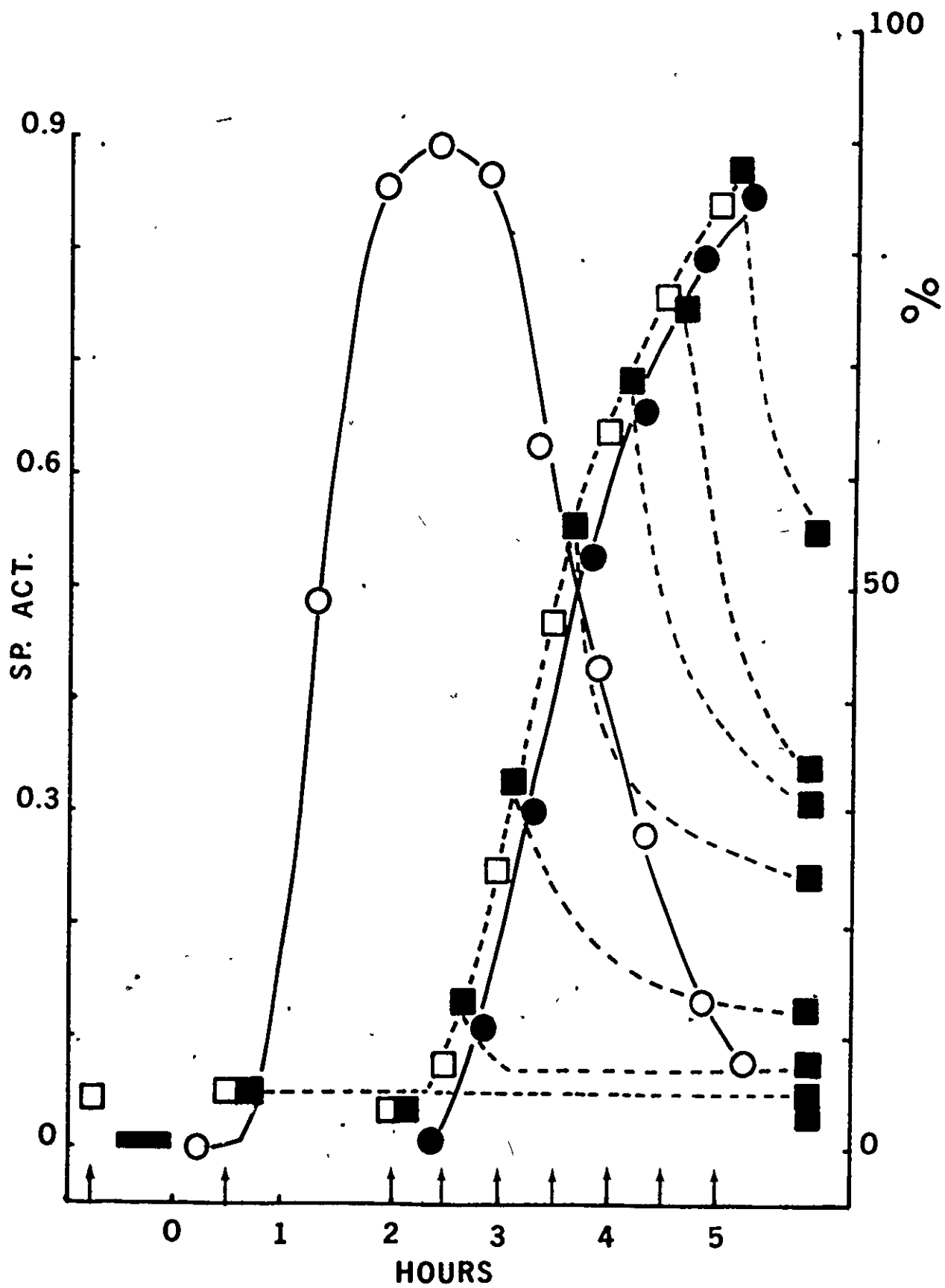
It was observed from the emergence data presented in Part I of the Results section of this thesis that a minimal fluence of  $77 \text{ J/m}^2$  (45 sec of UV exposure) was sufficient to

Figure 27.



A representative curve of the UV effect upon the expression of trehalase specific activity of germinating spores assayed immediately after irradiation or at 6 hours after heat activation. The arrows indicate the time of 8 separate applications of UV at a fluence of  $250 \text{ J/m}^2$ . The data presented in this figure is a composite curve of the data from Fig. 24 and Fig. 25. The dashed lines represent the kinetics of the decrease in trehalase specific activity after UV irradiation as determined in Fig. 26.

Symbols: ( ○ ) percent swelling of control spores  
 ( ● ) percent emergence of myxamobae from control spores  
 ( □ ) enzyme specific activity at the time of UV irradiation  
 ( ■ ) enzyme specific activity of irradiated spores immediately after UV and 6 hours after heat activation



reduce the percent emergence observed by 5 and 24 hours to below 1 percent. This same fluence also reduced the incorporation of radiolabelled uracil into TCA insoluble precipitates of germinating spores to a value about 10% of control uracil incorporation (Fig. 17, Part II). It is noted from the data presented in Table 8 that the expression of all three enzymes is equally inhibited at the lower total UV fluence.

Thus it appears that there is a range of UV fluences which can be employed to inhibit the emergence process as well as macromolecular synthesis that may be associated with this process without affecting the normal physiological responses of the spore. At UV fluences below  $77 \text{ J/m}^2$ , there is a graduated response in the final percent emergence that is attained by 5 and 24 hours after activation (Fig. 2, Part I). It is expected that at fluence below  $77 \text{ J/m}^2$  one should also observe graduated responses for the other processes in the spore that are affected by UV light. Ideally, in order to correlate the inhibition of a function with application of UV, the experiment should be carried out to at least a 10% level of inhibition (Sauerbier and Hercules, 1978). However, in a system such as Dictyostelium discoideum spore germination, the emergence phenomenon is an "all or none response." Thus it has been adequate to examine macromolecular responses of the spores on an "all or none" basis as well. Though the majority of the experiments employed  $250 \text{ J/m}^2$  UV light, it should be noted that a minimal UV fluence of about  $77 \text{ J/m}^2$  is sufficient to produce the same "all or none" response.



Table 8

Inhibition of Enzyme Expression in Spores Exposed to Minimal  
UV Fluences

Sample	B-glucosidase Specific Activity	Trehalase Specific Activity	Alkaline Phosphatase Specific Activity
Control			
0 Hr	0	1.0	4.2
5 Hr	95.4	18.4	40.2
Irradiated			
0 Hr	0	0.7	4.5
5 Hr	0	0.4	10.7

Spores were irradiated with  $77 \text{ J/m}^2$  (45 sec of UV exposure) which is the minimal UV fluence that reduces the percent emergence by 24 hours to below 1%. Enzyme assays were performed as described in the Materials and Methods section, and the enzyme specific activities are expressed as nmoles/min per mg protein, for purposes of comparison.

## DISCUSSION

Spores of Dictyostelium discoideum are unique in that they are more sensitive to ultraviolet radiation than are the vegetative cells (Liwerent and Pereira de Silva, 1975; Gillies et al., 1976; Ford and Deering, 1979; Hashimoto and Wada, 1980). The survival of UV irradiated spores of Dictyostelium discoideum depends both upon the ability of the myxamoebae to emerge from the spores as well as the ability of the amoebae, once emerged, to replicate and thus produce plaques on a bacterial lawn (Ford and Deering, 1979). In the experiments reported in this thesis, a small portion of spores that had not released amoebae by 5 hours after a UV treatment of less than  $77 \text{ J/m}^2$  (45 sec of UV exposure) appeared to be capable of releasing amoebae upon continued incubation. Such a delay in germination is not uncommon with irradiated spores (Zahl et al., 1939; Dimond and Duggar, 1940b, 1941); however it cannot be excluded that some of the amoebae observed by 24 hours after activation were due to cell division. Nevertheless, a comparison of data obtained from the emergence and viability studies (compare Fig. 2 and Fig. 6) indicates that a UV exposure time of 30 seconds (about  $50 \text{ J/m}^2$ ) is apparently required to reduce average survival to about 10 percent. Similar results for viability of irradiated cells of Dictyostelium

discoideum have been reported previously by Ford and Deering (1979). They obtained a  $D_{10}$  (10% survival) of  $70 \text{ J/m}^2$  for spores and  $160 \text{ J/m}^2$  for vegetative cells. In considering the greater sensitivity of the spore as compared to the vegetative cell, it is reasonable to postulate that functional repair enzymes may be lacking in the spore (Hashimoto and Wada, 1980). The fractionated exposure data would also indicate that little or no repair activity occurs between the periods of irradiation during spore germination (Fig. 3). The fact that irradiated spores also exhibited reciprocity suggests that a reduction of the UV fluence to lower intensities also does not enhance the efficiency of any possible repair (Fig. 4).

Since there is a slight shoulder in the survival curve of Dictyostelium discoideum spores (NC4), it would appear that they may not be completely devoid of some repair capability as would seem to be the case for the extremely radiation sensitive  $\gamma$ -sl3 mutant, which does not exhibit any shoulder in its vegetative cell UV survival curve. It is evident however that the repair capacity of the wildtype spores is considerably lower than that of the vegetative cells. Indeed, the radiation sensitivity of NC4 spores appears to be comparable to that of the vegetative Rad C mutants. Rad C mutants were found to have a  $D_{10}$  of  $50 \text{ J/m}^2$  (Kielman and Deering, 1980) which is very close to that observed for spores in this study. Furthermore, the vegetative cells of the Rad C mutants like the wildtype spores are equally as resistant to gamma rays as are the wildtype vegetative cells. It would thus not be

unreasonable to postulate that the repair deficiency of NC4 spores may be similar to that of the Rad C mutants which have been hypothesized to be deficient in endonuclease activity. Hashimoto and Wada (1980) suggest that it should be possible to determine whether the endonuclease activity is greater in amoebae than it is in spores. Hashimoto and Wada (1980) point out that more should be known about the cell cycle and its relationship to the dormant spore in order to adequately explain the radiation response of spores. It is possible to imagine that if spores are in the  $G_2$  stage of the cell cycle, then they may have a double complement of the genome and therefore possible be more radioresistant than if they were in the  $G_1$  stage of the cell cycle. However, though it has been widely observed that cells vary in their radiation response as they progress through the cell cycle, it has also been observed that the variations in radiation sensitivity are not in accord with the assumption that the  $G_2$  response should be the  $G_1$  response with twice the target number (Calkins, 1968). This has been observed for Dictyostelium. Survival curves following UV irradiation of diploids (constructed by the fusion of two radiation resistant haploids) differed only slightly from the wild type (NC4) haploid survival curve (Welker and Deering, 1978). Also, as presented in Table 2, the UV response of diploid spores is not significantly different from that of the haploid spores as far as the final percent emergence is concerned. Apparently, there are other factors which may influence the final shape of the survival

curve of diploid and haploid spores other than just their DNA complement. Another important aspect of the cell cycle which may be considered when evaluating radiation sensitivity is the time of the DNA synthetic periods. According to B. Lewin (1981) the synthesis of DNA, RNA and proteins are very characteristically regulated during the cell cycle. DNA and histones are only synthesized during the S phase and not during  $G_1$  or  $G_2$ , but cytoplasmic proteins and RNA are synthesized continually during  $G_1$ , S and  $G_2$ . Then during mitosis, the synthesis of DNA and RNA is turned off and protein synthesis is greatly reduced. Thus in eukaryotic cells the DNA of the chromosome may be made available for replication only during a specific portion of the cell cycle. Cleaver (1978) has suggested that the complex conformation of eukaryotic DNA may be partially responsible for the regulation of DNA synthesis from the point of view of the accessibility of the DNA to polymerases. Cleaver further points out that this may likewise account for the greater complexity of repair mechanisms for eukaryotic DNA. Though the size of the Dictyostelium genome has been reported as being only 11 times that of the E. coli genome (Sussman and Rayner, 1971) the structure of Dictyostelium chromatin has been described as similar to that seen in higher eukaryotes. (Bakke et al., 1978). Clark and Deering (1981) suggested that since the nuclear DNA in Dictyostelium occurs in a typical eukaryotic structure, it is also possible that impaired access to damaged DNA could account for the reduced repair seen in Rad C mutants and point out that this has been proposed as the basis for the repair defect in some Xeroderma pigmentosum cells (Mortelmans

et al., 1976). Van Etten (1969) has also asked the question "Is the spore DNA completely repressed so that even if all the components necessary to synthesize RNA and protein are present and active in ungerminated spores, the spore DNA cannot be transcribed?". Presently, nothing is known concerning the conformation of spore DNA of Dictyostelium discoideum.

It thus seems that there are a number of possibilities that may presently be considered in explaining the sensitivity of spores to UV light:

1. It is possible that spores are lacking in repair enzymes, such as the endonuclease activity which is deficient in the Rad C mutants. Such enzymes may not be synthesized until some time after emergence has occurred, thereby accounting for the increased resistance of the vegetative cells.
2. Repair enzymes may be present in the spores, but in a non-functional form.
3. Repair enzymes may be present in spores and in a functional form, but perhaps the DNA is not accessible to repair enzymes. One may postulate that DNA may become "poised" for replication only when cells are in the vegetative state, and perhaps the DNA is also amenable to repair at this time.

The third postulation itself is not unreasonable when one considers the observations of Gillies and co-workers (1975) and Hashimoto and Wada (1980). Amoebae that have just emerged from spores are still as sensitive to UV light as the spores. However, just before the first cell division, the UV resistance of vegetative cells reaches a maximum level. Finally

in the stationary phase of growth (6 hours after starvation) the UV sensitivity is again increased over the level found during the logarithmic phase of growth. It may be that during the logarithmic growth period cells pass through as S phase, prior to cell division, and perhaps repair synthesis can also occur at this time. This has been shown to occur in mouse L cells. Domon and Rauth (1973) irradiated mouse L cells during various stages of the life cycle and observed that the UV irradiated cells recovered from damage if they were allowed to undergo at least a first passage through the S phase. At present it is not possible to discern which hypothesis correctly explains why spores are more sensitive to UV light than vegetative cells.

In considering the effect of UV light on the spore germination process, it is evident that the sensitivity of spores appears to last until late spore swelling. After this time a "competence point" is reached, after which UV ( $250 \text{ J/m}^2$ ) no longer inhibits the emergence phenomenon. That fraction of cells which has passed the competence point is believed to have completed the events necessary for emergence. A minimal estimate for the competence point was determined to be approximately 30 minutes prior to emergence. Thus, all swollen spores that are within 30 minutes of emergence are committed to emerge even after the application of UV light.

Though UV definitely affects the final stage of germination (emergence) the initial stages of germination such as reversible activation, post-activation lag and swelling are

apparently unaffected. The slight delay in swelling observed after irradiation does not appear significant since all spores did reach the maximum swelling stage. The oxygen uptake which is a function of the swelling stage of germination is not inhibited by UV irradiation. The uptake of oxygen by spores however is inhibited by the addition of respiratory poisons such as cyanide and azide (Cotter et al., 1979). These poisons, as well as the incubation of spores under anaerobic conditions, also inhibited the swelling stage of germination. Swelling in Dictyostelium discoideum has thus been described as an aerobic energy requiring process. Cycloheximide, an inhibitor of cytoplasmic protein synthesis (Siegel and Sisler, 1964) was previously reported to reduce the cumulative volume of consumed oxygen by 15-20 percent in a 5 hour period of incubation and to block the release of myxamoebae from swollen spores (Cotter et al., 1979). This is not in contrast with the results obtained in this study. It was suggested that the apparent inhibition of oxygen uptake after 2 1/2 hours is a secondary effect of emergence suppression and is not a direct effect on the function of the electron transport system of the mitochondria (Cotter et al., 1979). The studies presented in this thesis demonstrate that UV irradiated spores exhibit similar O<sub>2</sub> accumulations as cycloheximide inhibited spores. Furthermore, a combination of both UV and cycloheximide was not additive. Finally, the application of UV at 2 hours after activation, also did not alter their initial rate of oxygen uptake; only the increased rate of O<sub>2</sub> accumulation,



presumably due to myxamoebae emergence was affected in these irradiated spores. Thus it can be presumed that UV light is not inhibiting the energy generating system of the spores.

UV also does not appear to interfere with the activation mechanism of spores since irradiated spores respond to the various means of chemical activation in the same manner as unirradiated controls. These activation treatments include the suspension of spores in such agents as DMSO, ethylene glycol, urea, and methanol for various periods of time (see Materials and Methods). Irradiated spores which have been activated may also be deactivated by incubation in 0.25 M sucrose. It is thus expected that irradiated spores should also respond to other means of deactivation such as by incubation at 0 C (Cotter and Raper, 1968a) or by incubation in respiratory poisons (Cotter et al., 1979). In addition to the phenomenon of exogenously induced spore activation, the auto-activation mechanism of Sgl spores is not inhibited by UV, although the kinetics of germination may be somewhat altered.

The agents which have been observed to exogenously activate spores have been noted by Cotter (1975) to have the common property of being protein denaturants. A multistate model of activation has been proposed by Cotter (1973, 1975) to explain the various activation-deactivation phenomenon. Thermodynamic estimations by Cotter (1973) support the idea of a protein as being the target site for activation in the membrane. However, other explanations as to the activation mechanism have also been offered. Bacon and Sussman (1973) suggested that heat

treatments for example may result in the activation of protein synthesizing machinery of the spores, and blockage of this early protein synthesis results in spore deactivation. Hohl (1976) postulated that heat activation allows ATP synthesis required for the synthesis of tryptophane, which in turn is needed by protein synthetic machinery for the formation of a spore swelling enzyme. The recent UV studies would tend to support the idea that although energy may be involved, de novo protein synthesis may not be required for spore activation and deactivation. Activation and deactivation of spores appear to occur despite UV irradiation and the apparent inactivation of DNA template functions. The fact that UV irradiation does not inhibit autoactivation of Sgl spores suggests that the autoactivation mechanism is also not dependent on de novo RNA and protein synthesis.

The idea that the early events in spore germination may be a result of preformed messenger RNA has been proposed previously in other systems where actinomycin D was used as an RNA synthesis inhibitor. For example, actinomycin D has been shown to inhibit mRNA synthesis but not its function (protein synthesis) in organisms such as Bacillus species (Levinthal et al., 1962; Kobayashi et al., 1965). In these Bacillus spore systems it was observed that actinomycin D had no effect on the initial stages of germination (loss of heat resistance, loss of refractability) but completely inhibited protein synthesis in the latter stages. Sussman and Halvorson (1966) concluded that in spores where this phenomenon is observed,

the spores may be devoid of stable mRNA and that protein synthesis occurs only following mRNA synthesis.

In considering the phenomenon of "outgrowth" in bacterial spores Sussman and Halvorson (1966) have suggested two possibilities:

1. The dormant spore contains stable mRNA molecules for the synthesis of vegetative enzymes.
2. One of the early steps in germination and outgrowth is the synthesis of vegetative mRNA.

Presently, for the majority of spore systems, the second hypothesis is favored (Sussman and Halvorson, 1966). The UV data would support the idea that the spores of Dictyostelium discoideum are also among those for which the second hypothesis is favored, although the possible existence of some stable mRNA in the dormant spore cannot be excluded.

Thus from the results of part I of this thesis it may be concluded that spores of Dictyostelium discoideum are considerably UV sensitive and do not appear to be capable of recovering from UV-induced damage, at least after fluences above  $77 \text{ J/m}^2$ . A UV fluence as high as  $250 \text{ J/m}^2$  however, will not inhibit the emergence of myxamoebae from spores, if it is applied after a "critical time" in the germination sequence which we have defined as the "competence point". It is also evident that the initial events of germination (reversible activation, swelling and respiration) occur independently of the UV sensitive site(s), but the final event of germination (emergence) is dependent on UV sensitive functions.

Since UV light has been observed to inhibit the emergence phenomenon in spores and since the emergence phenomenon has been postulated to be dependent on macromolecular synthesis, the second portion of this thesis examined the effect of UV on macromolecular synthesis during spore germination.

It is known that it is DNA synthesis that is most inhibited in UV irradiated systems (see literature review), followed by the inhibition of RNA and protein synthesis. The synthesis of all three macromolecular species during spore germination has been examined by monitoring the incorporation of radio-labelled precursors during the 5 hour incubation period. Contrary to earlier results of Yagura and Iwabuchi (1976) DNA synthesis as measured by the incorporation of radiolabelled thymidine could not be detected during the 5 hour incubation period of spore germination. Furthermore, UV irradiated spores also did not exhibit any unscheduled DNA repair synthesis, which has been shown to occur in some irradiated systems. Such synthesis has been described as not being of the normal semi-conservative type of DNA synthesis (Pettijohn and Hanawalt, 1964). It has been discussed that DNA synthesis may not be occurring during the spore germination process, although it should be pointed out that any definite conclusions in this regard have been hampered by our lack of knowledge about the stage of the cell cycle which occurs during sporulation. The fact that DNA synthesis may not be required for the spore germination process in Dictyostelium discoideum has been postulated earlier by Cotter (Ph.D. Thesis, 1967).

Cotter reports that there is no apparent doubling of the DNA content during the spore germination process as the DNA content of haploid vegetative cells is approximately equivalent to that of haploid dormant spores. The DNA content of the diploid cells on the other hand was found to be about twice that of the haploid cells (Cotter, Ph.D. thesis, 1967). Presently there are no other reports in the literature of DNA synthesis occurring during the germination of Dictyostelium discoideum spores.

Although the spores of Dictyostelium discoideum do not appear to be amenable to the study of DNA synthesis, it is apparent the much RNA and protein synthesis is occurring during the spore germination process as has been shown by a number of researchers (Cotter and Raper, 1970; Bacon and Sussman, 1973; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977, 1978; Cotter et al., 1979). It is evident from the studies presented in part II of this thesis, that RNA synthesis, as measured by the incorporation of radiolabelled uracil into TCA insoluble precipitates, is severely inhibited by UV irradiation. A fluence of  $250 \text{ J/m}^2$  is sufficient to reduce the incorporation level to about 10 percent that of control levels. A UV fluence of about  $47 \text{ J/m}^2$  however was found to be sufficient to reduce the final percent emergence to below 1 percent. Apparently, this same minimal fluence is equally effective in reducing the incorporation of uracil to about 10 percent of the control levels. This indicates that there is a range of UV fluences which can be applied to spores which

results in relatively the same amount of RNA synthesis. It is possible that the 10 percent level of incorporation observed at the range of fluences applied is only residual incorporation into prematurely terminated transcripts (Giorno, 1979a, b). A minimal fluence which is required to prevent emergence may result in a low level of RNA synthesis which is not further reduced by the application of higher UV fluences, as the threshold inhibition has already been achieved. It may be possible to observe a gradual increase in the rates of RNA synthesis in spores that are irradiated with lower UV fluences (below  $77 \text{ J/m}^2$ ). However, at such sublethal fluences, the emergence of spores is no longer suppressed (see Fig. 2).

Although the particular species of RNA affected by UV has not been examined in this thesis (and is in fact the topic of another thesis) it is not unreasonable to postulate that the presence of unrepaired UV-induced lesions in the DNA template of the spore is inhibiting the transcription process (see literature review). This would imply that the synthesis of RNA is being affected in UV irradiated spores such that the messages encoding for specific emergence proteins are prematurely terminated. Evidence has just recently been obtained demonstrating that there is a decrease in the level of mRNA in spores of Dictyostelium discoideum following UV irradiation (Hamer, personal communication). However, at present, since the RNA also absorbs UV light, it must be considered as a potential site for UV-induced lesions. This includes the possibility of a direct effect of UV on such

species of RNA as the mRNA or tRNA. In general though, it has been shown that the translation process and functions that are related to translation are less affected by UV light than those functions attributed to the transcription process (see literature review). This is not unreasonable when one considers that only one unrepaired lesion may be required to inactivate DNA template function (Caillet-Fauquet et al., 1977; Ali and Sauerbier, 1978). Ponta and co-workers (1979) have shown that a unit of mRNA template function is more sensitive to UV than a unit of DNA template function; however, there are no doubt many more copies of messenger RNA encoding for a particular function than DNA in any particular cell. Firtel (1972) has examined the number of genes expressed preferentially during development by measuring the RNA sequences present at various stages with hybridization techniques. The level of mRNA for a few developmentally regulated genes has been calculated, and it has been estimated that the mRNAs for some developmentally regulated proteins must be present at or above 80 copies per cell (Loomis, 1978). This at present seems to be the best argument that the results observed in this study are not primarily due to inactivation of translation functions as opposed to transcription functions.

The inhibition of protein synthesis in UV irradiated spores has been examined from two points of view in this thesis, i.e., by the incorporation of radiolabelled protein precursors and by the examination of the expression of specific enzymes during the spore germination process. It was observed that

protein synthesis, as measured by the incorporation of radio-labelled leucine into TCA insoluble precipitates, is inhibited in UV irradiated spores. This inhibition of protein synthesis is most probably due to the inhibition of RNA synthesis as described above. According to the incorporation data, the bulk of protein synthesis, like RNA synthesis, occurs at some time just prior to the emergence of myxamoebae. This increase in incorporation of RNA and protein precursors has also been previously shown to be inhibited in spores incubated in the presence of puromycin, edeine, and cycloheximide (Cotter and Raper, 1970; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977, 1978; Cotter et al., 1979). The exact time of commencement of protein synthesis as judged by the incorporation of labelled precursors in spores and by the appearance of polysomes begins at the initiation of spore swelling and then increases rapidly at later stages. It is possible that the incorporation data is a reflection of spore permeability rather than an accurate indication of actual protein synthesis occurring in the cell (Ceccarini, 1967). Indeed, swollen spores appear to be permeable to label, but it is not certain if dormant spores are equally permeable. However, it has been shown that the peptide antibiotic, edeine, will enter spores during heat activation (Tisa, Ms. Thesis, 1979), and it is thus thought that exogenous amino acids should also be able to enter the dormant spores. It has already been suggested that incorporation data can only provide a limited indication of the actual protein synthesis occurring during germination without a knowledge of



precursor pools (Giri and Ennis, 1977). However, the comparison of the uptake and incorporation data in this work would suggest that incorporation is reflecting actual protein synthesis, at least during the swelling and emergence stages of spore germination.

In this study, it was observed that cycloheximide, a protein synthesis inhibitor, was having an effect on the incorporation of radiolabelled uracil into germinating spores. This has been previously observed for Dictyostelium discoideum by Giri and Ennis (1977). The fact that cycloheximide may cause some inhibition of RNA synthesis has also been observed for Polysphondylium pallidum microcysts by O'Day (1976) and in numerous other organisms (Kloet, 1966; Timberlake and Griffen, 1972, 1974). Researchers have postulated that perhaps some RNA synthesis is dependent on the continued synthesis of a factor or protein, although few definite conclusions have been made as to the exact nature of the substance that is synthesized.

The data presented in Fig. 18 (Part II) suggest that transcription of mRNA required for the formation of "emergence proteins" is not completed in the presence of cycloheximide. Spores which are irradiated after cycloheximide has been removed (at a time when emergence is almost completed in control spores) are still inhibited from releasing amoebae. If transcription had been completed in the presence of cycloheximide (as was previously postulated) then UV irradiation, after cycloheximide removal, should no longer prevent the emergence

phenomenon. Remember that UV irradiation after a critical point, which we have defined as the competence point, no longer inhibits the emergence in spores that have passed this point in the germination sequence (Fig. 9a, Part I). Since transcription is apparently not completed in the presence of cycloheximide, it may be possible that transcription is indirectly being affected by cycloheximide, perhaps by a mechanism that is not unlike that suggested by Kloet (1966) for yeast or by Timberlake (1974) for Achyla (see results Part II, section 2). Thus it is possible that continued RNA synthesis does require the formation of a factor (RNA polymerase?) and the inhibition of protein synthesis inhibits the continued synthesis of this "labile" specific factor. This hypothesis may explain the decrease in the rate of uracil incorporation into RNA of cycloheximide treated spores, as well as the fact that cycloheximide treated spores do not release amoebae if they are irradiated within 1 hour after the cycloheximide has been removed.

As was observed previously, the final percent emergence achieved appears to be proportional to the delay in time of UV application, in this case, after cycloheximide removal. Since the accumulation of the three enzymes, B-glucosidase, alkaline phosphatase and trehalase have been shown to occur prior to or concomitantly with emergence, it is expected that a proportional relationship should also be observed between enzyme specific activity and delay in time of UV application, following removal of cycloheximide.

Part II of the results of this thesis has examined the effect of UV light on the synthesis of the three general macromolecular classes, DNA, RNA and protein, as reflected by the uptake and incorporation data. Part III of the results of this thesis has been concerned mainly with the effects of UV light on protein synthesis as measured by the expression of specific enzymes. The enzymes B-glucosidase, alkaline phosphatase and trehalase, have all previously been shown to exhibit significant increases in specific activity during vegetative development (see Loomis, 1975, for a review). RNA and protein synthesis inhibitors can inhibit the "stage specific" expression of these three enzymes during the spore germination process (Cotter and Raper, 1970; Cotter *et al.*, 1979; Tisa and Cotter, 1980a, b). All three enzymes studied here appear to be "coordinately controlled" despite the fact that they are apparently located at different sites in the cell. B-glucosidase, an acid hydrolase is believed to be contained in the lysosomes of the cells (Ashworth and Quance, 1972; Oohata, 1976). Alkaline phosphatase may be associated with the plasma membrane (Parish and Pelli, 1974) or the cell surface (Crean and Rossomondo, 1977) and its activity has also been associated with contractile vacuoles (Quivigar *et al.*, 1978, 1980). The trehalase enzyme has not as of yet been localized in the cell. Initial experiments with 20,000 x g and 105,000 x g supernatants indicated that it may not be membrane bound (Cotter and Raper, 1970). However, trehalase (unlike B-glucosidase and alkaline phosphatase) may be located in such as area of the

cell where it can readily be released to the medium. It has been demonstrated that the internal drop in trehalase activity may be attributed to a loss of trehalase to the external medium (Chan, personal communication). The fact that UV irradiation inhibits the expression of B-glucosidase, alkaline phosphatase and trehalase, now provides further evidence that some macro-molecular event, which is common to all three enzymes, is being affected. The critical event(s) which is inhibited by UV, that affects the expression of these three enzymes as well as the emergence of myxamoebae from spores, can still only be postulated. The fact that the event is UV sensitive is suggestive of the involvement of the nucleic acids with DNA being the most likely target. This in turn may implicate a "de novo" synthesis of these enzymes during spore germination.

Support for the idea of a UV associated inhibition of "de novo" enzyme expression has been found in numerous systems. UV light is known as a potent inhibitor of induced enzyme synthesis in bacteria and yeast. Such induced synthesis requires transcription from a DNA template. For example, when the induced synthesis of arginase was measured in several yeast strains after UV, there was an exponential relationship between a decrease in enzyme activity and an increase in the ultraviolet fluence (Gocke and Kiefer, 1977). Also, UV irradiation of E. coli suppressed almost completely, the ability to synthesize induced B-galactosidase and this synthetic ability was restored largely by photoreactivation (Kameyama and Novelli, 1962). It was found by these researchers that the

induced synthesis of B-galactosidase in E. coli was more sensitive to ultraviolet than general protein synthesis. In another system, Bacillus cereus, the UV sensitivity of the induced synthesis of penicillinase was seen to vary with the time of exposure to UV after induction (Torriana, 1956). The behavior of this system was consistent with a model that assumed that "UV blocks the synthesis of new enzyme forming template, but does not affect the synthetic activity of templates already in existence at the time of irradiation" (Kameyama and Novelli, 1962). Similarly in Pseudomonas effusa, the induced synthesis of the enzyme catechol oxygenase in the resting cells, stopped at about 8 minutes after the application of UV light (Atsuta et al., 1964). The time period of 8 minutes was believed to be the half life of the messenger RNA for catechol enzymes, and thus they concluded that UV irradiation did not inhibit the synthesis of catechol oxygenase immediately after catechol (the inducer) removal, but it was the further increase in enzyme activity after this time that was inhibited. They concluded that the inhibition of the induced formation of catechol oxygenase by UV is due to an inhibition of further mRNA transcription from DNA, rather than due to an effect of UV on the coding process of amino acids on ribosomes (Atsuta et al., 1964).

In the case of Dictyostelium discoideum there has also been some work which examined the effect of radiation on enzyme synthesis in vegetative cells. Cleveland and Deering (1976) studied the effects of gamma irradiation on the specific

activity levels of the enzymes UDP-glucose pyrophosphorylase and alkaline phosphatase. They found that there was a delay in the appearance of peak pyrophosphorylase and peak alkaline phosphatase specific activity and that this was closely correlated with a morphogenetic delay. The repair proficiency of the vegetative cells of Dictyostelium discoideum has already been discussed (see literature review) and the fact that the enzyme specific activities were only delayed rather than completely inhibited at the lower gamma radiation doses further demonstrates the repair proficiencies of the vegetative cells. The reduction in enzyme activity was not due to direct radiation inactivation of the enzyme since the specific activities of the enzymes were unchanged immediately after irradiation (Cleveland and Deering, 1976). The fact that a loss in specific activity was observed only after a delay suggested to Cleveland and Deering that this was consistent with the idea of a ~~slow~~ degradation or inactivation of enzyme already present, combined with a failure to synthesize de novo more enzyme. Cleveland and Deering could not conclude from their work whether the radiation-induced morphogenetic delay resulted from the delays in appearance of developmental enzymes, or whether some other effect of the gamma radiation interfered with developmental control. Although gamma irradiation has been associated with transcription terminating effects in other systems (Kanazir, 1969) the effects have not been so well characterized as for UV irradiation. Thus far, it has not yet been determined if enzymatic delays due to UV light

are associated with morphogenetic delays of UV irradiated vegetative cells of Dictyostelium discoideum. However, the work of Firtel and co-workers (1973) using actinomycin D and daunomycin to inhibit enzyme expression, is very comparable to Cleveland and Deering's work with regards to the timing of the supposed transcriptive period during development. Alkaline phosphatase and UDP-galactosidase were among the enzymes observed by Firtel and co-workers, who concluded in their studies that the expression of these enzymes was transcriptionally regulated.

Thus the examination of enzyme expression does appear to be a useful method with which to study cellular protein synthesis and the effects of physical and chemical inhibitors on gene expression. However, though the expression of specific "marker" enzymes may give an indication of what may be happening to enzymes in general, which are expressed at this time, it is difficult to correlate specific enzyme activity with a particular morphogenetic function. The inhibition of a particular enzyme, along with the inhibition of a particular function, does not prove that the expression of that enzyme is critical to the function. However, if the function occurs despite the fact that the expression of the particular enzyme is inhibited, this may suggest that the enzyme in question is not itself critical to the function.

In a general review on protein synthesis in spores Lovett (1976) discusses the fact that there is abundant evidence for increases in specific activities during spore germination,

but none of the increased enzyme activities have been shown to be necessary for the success of germination (Tuve'sson, 1967; Tisdale and Debusk, 1970; Ohmori and Gottlieb, 1965). Lovett points out that in the few cases where the sudden appearance of an enzyme has been implicated in spore germination, the enzyme has been shown to be preformed and either activated or released at germination (Sussman, 1966; Van Assche *et al.*, 1972; Page and Stock, 1972; Silverman *et al.*, 1974). It is concluded by Lovett that "the large variety of proteins assembled at very early times in spore germination, suggest that the probability of finding a single essential protein may be low".

The role of these three enzymes, B-glucosidase, alkaline phosphatase and trehalase during the spore germination process is still obscure. Their apparent expression just prior to emergence might suggest that any one or all three might have some essential function for emergence. The recent establishment of a "competence point" has however placed this possibility under question. It is believed that any spore that has passed the competence point, can no longer be inhibited by UV irradiation, and is indeed committed to emerge (Demsar and Cotter, in press). The data presented in this study indicate that the expression of B-glucosidase and trehalase occur after the competence point and may thus be non-essential to emergence. Amoebae in the population are still capable of emerging for some time even though enzyme accumulation has ceased shortly after UV. However, there is a low level of alkaline phosphatase



activity which is apparently not inhibited by UV. This low alkaline phosphatase activity (about 2 fold over 0 hour values) has also been previously observed in spores inhibited at the maximally swollen stage by cycloheximide, puromycin and edeine (Tisa and Cotter, 1979). Recent evidence has been presented which demonstrates that there is a reversible heat activation of membrane-bound alkaline phosphatase (Mohan Das and Weeks, 1980). Das and Weeks suggest that the increase in alkaline phosphatase activity may be due to an unmasking of pre-existing enzyme. It is not known whether this early expression of a low level of alkaline phosphatase has any role in the emergence process. The bulk of alkaline phosphatase activity, like B-glucosidase and trehalase, appears to be expressed after the competence point.

Though the competence data is only suggestive, it does indicate that enzymes which are expressed very late in the germination sequence may not actually be among those which are critical to emergence, even though they may provide some necessary function to emerged vegetative amoebae. It is probable that the enzymes which are critical to the emergence process are actually those which are synthesized prior to the competence point. However, it is also must be that the synthesis of the critical enzymes is not completed at least until the time that the competence point has been reached. Thus though it is still possible that some preformed enzymes may serve a critical function to the emergence process, such as cellulase for example (Jones et al., 1979) there is a definite

requirement for some de novo enzyme synthesis. This required enzyme synthesis is likely to occur during the early or middle portion of spore swelling. The fact that there is some early protein synthesis occurring prior to competence is reflected in the radiolabelled leucine incorporation data that has been observed in this study (Fig. 20) and in other studies as well (Bacon and Sussman, 1973; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977, 1978).

The data presented in Part III of the Results of this study demonstrate the difficulty of attributing the occurrence of certain developmental events to prior enzyme expression. Although it has been shown that there is a protein synthetic requirement for the emergence function of Dictyostelium discoideum spores, the actual enzymatic requirements have yet to be defined, since the particular class of proteins involved in emergence remains obscure. Indeed it might be quite a formidable task, or possibly even a stroke of good fortune to fall upon the exact enzymes which are involved, when one considers the number of genes that have been estimated for the Dictyostelium genome. Results of Firtel (1972) suggest that about 16,000 sequences of 1000 bases (sufficient to be a gene) are transcribed during development (Loomis, 1978). However it has also been discussed by Loomis (1978) that of these genes, possibly only 400 or so are actually specific and essential for development.

The fact that regulation may be occurring at the level of mRNA synthesis has been postulated previously for Dictyostelium

discoideum vegetative cells (Firtel et al., 1973) and also for events which occur during the spore germination process (Dowbenko and Ennis, 1980). The idea that "commitment" to a particular phase of development occurs at the level of mRNA has also been postulated in other systems, based on data which is comparable to that obtained in the studies presented here. For example, the inhibitor actinomycin D, was used to study the sporulation process in Bacillus subtilis, with respect to a number of events which were sequentially expressed at particular times during the sporulation process. It was concluded that commitment occurred at a different point for each particular event, and that each point of commitment was associated with an apparently stable species of messenger RNA (Al-Shaikley et al., 1976). According to the kinetics of enzyme inactivation for the enzymes observed during Dictyostelium discoideum spore germination, it would appear that the messenger RNA encoding for these three enzymes may be relatively shortlived (appears to be less than 15 minutes). The existence of short-lived mRNA has recently been confirmed for Dictyostelium discoideum spores (Hamer, personal communication). On the other hand, emergence appears to continue for at least 30 to 45 minutes after the application of UV light. Thus, the particular species of enzyme that is critical to the emergence function must either be synthesized earlier than those observed in this study, or the half life of its messenger RNA might be somewhat longer than that of the mRNA coding for these three particular enzymes. If the half lives of some of the mRNA

encoding for certain proteins were found to be 30 - 45 minutes long, then these enzymes should accumulate in a similar manner as the percent emergence, following UV irradiation. Such enzymes may thus also be considered among those which have a potential to be essential for the emergence function.

Thus far, such enzymes which are supposedly expressed early in the germination sequence as a results of de novo synthesis have not yet been identified. The separation of proteins by polyacrilamide gel electrophoresis has been a useful method as far as indicating the presence of "newly synthesized" proteins during spore germination (Dowbenko and Ennis, 1980; Alton and Lodish, 1977a, b). However, the identification of the characteristic spots of such gels would also be a formidable task. It has been suggested that it is possible that some perhaps essential enzymes may occur in such low concentrations so as not to be detectable by such methods (Dowbenko and Ennis, 1980). The UV technique presented in this thesis, may however be helpful in at least providing information as to the timing of the expression of possibly critical enzymes. It has been pointed out by Scandolios (1980) that:

"The analysis of enzyme expression provides a reasonable and promising approach towards the regulation of gene expression providing we accept the underlying assumption that the characteristics of a given cell at different stages of development are functions of the protein molecules existing in those cells."

In conclusion, there are three general levels of regulation which are currently recognized; transcriptional control, pertaining to the synthesis and degradation of mRNA; translational control, pertaining to the processing and utilization

of mRNA; and post-translational control; pertaining to the processing and modification of protein molecules following peptide synthesis (Scandolios, 1980). Though the UV data is suggestive of control at the level of mRNA transcription, the work presented in this thesis does not allow one to discern to what degree any or all three levels of control are evident during spore germination in Dictyostelium discoideum. It is attractive at present to postulate that the emergence functions, may be events which are transcriptionally regulated during germination. The question of regulation in Dictyostelium will no doubt provide a source of intrigue to researchers for yet a number of years to come.



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VITA AUCTORIS

Irene Helen Demsar

Born: May 13, 1954, Windsor, Ontario, Canada

Parents: Mr. and Mrs. John Demsar

Education: Graduated with honours from F. J. Brennan High School, Windsor, Ontario in the spring of 1973.

Received a Bachelor of Science in Biology at the University of Windsor, Windsor, Ontario in the spring of 1977.